



US006372453B1

(12) **United States Patent**
Klein et al.

(10) **Patent No.:** **US 6,372,453 B1**
(45) **Date of Patent:** ***Apr. 16, 2002**

(54) **NEURTURIN RECEPTOR**

(75) Inventors: **Robert D. Klein**, Palo Alto; **Arnon Rosenthal**, Burlingame, both of CA (US)

(73) Assignee: **Genetech, Inc.**, South San Francisco, CA (US)

(*) Notice: This patent issued on a continued prosecution application filed under 37 CFR 1.53(d), and is subject to the twenty year patent term provisions of 35 U.S.C. 154(a)(2).

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **08/802,805**

(22) Filed: **Feb. 18, 1997**

(51) **Int. Cl.**⁷ **C12P 21/06**; C12N 1/20; C12N 15/00; C07H 21/02

(52) **U.S. Cl.** **435/69.1**; 435/69.7; 435/70.1; 435/71.1; 435/252.3; 435/254.11; 435/320.1; 435/325; 530/300; 530/350; 530/827; 536/23.1; 935/6; 935/22; 935/66; 935/109

(58) **Field of Search** 536/23.5, 23.1; 435/6, 69.1, 320.1, 325, 69.7, 70.1, 71.1, 252.3, 254.11; 935/6, 22, 66, 109; 530/300, 350, 827

(56) **References Cited**

U.S. PATENT DOCUMENTS

6,025,157 A * 2/2000 Klein et al.

FOREIGN PATENT DOCUMENTS

WO	WO 91/06667	5/1991
WO	WO 93/06116	4/1993
WO	WO 97/44356	11/1997

OTHER PUBLICATIONS

Jing, S. et al., *Cell*, 85(7):1113–24, 1996.*
The WashU–Merck EST Project, Accession No. R02249, 1995.*
Andres et al., “Expression of two novel eph–related receptor protein tyrosine kinases in mammary gland development and carcinogenesis” *Oncogene* (erratum) 9:2431 (1994).
Andres et al., “Expression of two novel eph–related receptor protein tyrosine kinase in mammary gland development and carcinogenesis” *Oncogene* 9:1461–1467 (1994).
Arenas et al., “GDNF Prevents Degeneration and Promotes the Phenotype of Brain Noradrenergic Neurons in Vivo” *Neuron* 15:1465–1473 (1995).
Baloh et al., “TrnR2, a Novel Receptor That Mediates Neurturin and GDNF Signaling through Ret” *Neuron* 18(5):793–802 (May 1997).
Beck et al., “Mesencephalic dopaminergic neurons protected by GDNF from axotomy–induced degeneration in the adult brain” *Nature* 373:339–341 (1995).
Bennett et al., “Cloning and Characterization of HTK, a Novel Transmembrane Tyrosine Kinase of the EPH Sub-

family” *Journal of Biological Chemistry* 269(19):14211–14218 (1994).
Berkemeier et al., “Neurotrophin–5: A Novel Neurotrophic Factor That Activates trk and trkB” *Neuron* 7:857–866 (Nov. 1991).
Buj–Bello et al., “GDNF Is an Age–Specific Survival Factor for Sensory and Autonomic Neurons” *Neuron* 15:821–828 (1995).
Buj–Bello et al., “Neurturin responsiveness requires a GPI–linked receptor and the Ret receptor tyrosine kinase” *Nature* 387(6634):721–724 (Jun. 12, 1997).
Cash et al., “Parkinson’s disease and dementia: Norepinephrine and dopamine in locus ceruleus” *Neurology* 37:42–46 (1987).
Chan–Palay et al., “Alterations in Catecholamine Neurons of the Locus Coeruleus in Senile Dementia of the Alzheimer Type and in Parkinson’s Disease With and Without Dementia and Depression” *The Journal of Comparative Neurology* 287:373–392 (1989).
Davis et al., “Released form of CNTF receptor α component as a soluble mediator of CNTF responses” *Science* 259:1736–1739 (1993).
Durbec et al., “GDNF signalling through the Ret receptor tyrosine kinase” *nature* 381:789–793 (1996).
Hefti, F., “Nerve Growth Factor Promotes Survival of Septal Cholinergic Neurons After Fimbrial Transections” *J. of Neuroscience* 6(8):2155–2162 (Aug. 1986).
Henderson et al., “GDNF: A Potent Survival Factor for Mononeurons Present in Peripheral Nerve and Muscle” *Science* 266:1062–1064 (1994).
Heumann, R., “Regulation of the Synthesis of Nerve Growth Factor” *J. Exp. Biol.* 132:133–150 (1987).
Hillier, L. et al., “The WashU–Merck EST Project” (Accession No. R02249) (1995).
Hirano, A., “Cytopathology of Amyotrophic Lateral Sclerosis” *Advances in Neurology: Amyotrophic Lateral Sclerosis and Other Motor Neuron Diseases*, Lewis P. Rowland, Raven Press, Ltd., Chapter 8, vol. 56:91–101 (1991).
Hirsch et al., “Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson’s disease” *Nature* 334:345–348 (1988).
Hornykiewicz, O., “Neurochemical Pathology and the Etiology of Parkinson’s Disease: Basic Facts and Hypothetical Possibilities” *Mt. Sinai J. Med.* 55:11–20 (1988).
Ikeda et al., “Specific expression of the ret proto–oncogene in human neuroblastoma cell lines” *Oncogene* 5:1291–1296 (1990).

(List continued on next page.)

Primary Examiner—Patricia A. Duffy
(74) *Attorney, Agent, or Firm*—Knobbe, Martens, Olson & Bear, LLP

(57) **ABSTRACT**

NTNR α , NTNR α extracellular domain (ECD), NTNR α variants, chimeric NTNR α (e.g., NTNR α immunoadhesin), and antibodies which bind thereto (including agonist and neutralizing antibodies) are disclosed. Various uses for these molecules are described, including methods to modulate cell activity and survival by response to NTNR α –ligands, for example NTN. by providing NTNR α to the cell.

37 Claims, 21 Drawing Sheets

OTHER PUBLICATIONS

- Jing et al., "GDNF-Induced Activation of the Ret Protein Tyrosine Kinase Is Mediated by GDNFR- α , a Novel Receptor for GDNF" *Cell* 85:1113-1124 (1996).
- Jing et al., "GFR α -2 and GFR α -3 Are Two New Receptors for Ligands of the GDNF Family" *Journal of Biological Chemistry* 272(52):33111-33117 (Dec. 26, 1997).
- Kaisho et al., "Cloning and expression of a cDNA encoding a novel human neurotrophic factor" *FEBS Letters* 266(1, 2):187-191 (Jun. 1990).
- Kearns et al., "GDNF protects nigral dopamine neurons against 6-hydroxydopamine in vivo" *Brain Research* 672:104-111 (1995).
- Klein et al., "A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor" *Nature* 387(6634):717-721 (Jun. 12, 1997).
- Koke et al., "High-Level Expression in *Escherichia coli* and Rapid Purification of Phosphatidylinositol-Specific Phospholipase C from *Bacillus cereus* and *Bacillus thuringiensis*" *Prot. Express, Purification* 2:51-58 (1991).
- Kotzbauer et al., "Neurturin, a relative of glial-cell-line-derived neurotrophic factor" *Nature* 384:467-470 (1996).
- Lai et al., "An Extended Family of Protein-Tyrosine Kinase Genes Differentially Expressed in the Vertebrate Nervous System" *Neuron* 6:691-704 (May 1991).
- Lee et al., "Glycosyl-Phosphatidylinositol-anchored or integral membrane forms of CD14 mediate identical cellular responses to endotoxin" *Proc. Natl. Acad. Sci. USA* 90:9930-9934 (1993).
- Leibrock et al., "Molecular Cloning and Expression of Brain-derived Neurotrophic Factor" *Nature* 341:149-152 (Sep. 14, 1989).
- Lin et al., "GDNF: A Glial Cell Line-Derived Neurotrophic Factor for Midbrain Dopaminergic Neurons" *Science* 260:1130-1132 (1993).
- Lindsay and Yancopoulos, "GDNF in a Bind with Known Orphan: Accessory Implicated in New Twist" *Neuron* 17:571-574 (1996).
- Maisonpierre et al., "Ehk-1 and Ehk-2: two novel members of the Eph receptor-like tyrosine kinase family with distinctive structures and neuronal expression" *Oncogene* 8:3277-3288 (1993).
- Maisonpierre et al., "Neurotrophin-3: A Neurotrophic Factor Related to NGF and BDNF" *Science* 247:1446-1451 (Mar. 23, 1990).
- Marcyniuk et al., "The Topography of Cell Loss from *Locus Caeruleus* in Alzheimer's Disease" *J. Neurol. Sci.* 76:335-345 (1986).
- Micanovic et al., "Selectivity of the cleavage/attachment site of phosphatidylinositol-glycan-anchored membrane proteins determined by site-specific mutagenesis at Asp-484 of placental alkaline phosphatase" *Proc. Natl. Acad. Sci. USA* 87:157-161 (1990).
- Moore et al., "Renal and neuronal abnormalities in mice lacking GDNF" *Nature* 382:76-79 (1996).
- Moran et al., "Glycophospholipid membrane anchor attachment. Molecular analysis of the cleavage/attachment site" *Journal of Biological Chemistry* 266(2):1250-1257 (Jan. 15, 1991).
- Oppenheim et al., "Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF" *Nature* 373:344-346 (1995).
- Pasquale et al., "Identification of a Developmentally Regulated Protein-Tyrosine Kinase by using anti-phosphotyrosine Antibodies to Screen cDNA Expression Library" *Proc. Natl. Acad. Sci. USA* 86:5449-5453 (1989).
- Pasquale, E., "Identification of chicken embryo kinase 5, a developmentally regulated receptor-type tyrosine kinase of the Eph family" *Cell Regulation* 2:523-534 (1991).
- Pichel et al., "Defects in enteric innervation and kidney development in mice lacking CDNF" *Nature* 382:73-76 (1996).
- Poulsen et al., "TFG β 2 and TGF β 3 are potent survival factors for midbrain dopaminergic neurons" *Neuron* 13(5):1245-1252 (Nov. 1994).
- Pugin et al., "Lipopolysaccharide activation of human endothelial and epithelial cells is mediated by lipopolysaccharide-binding protein and soluble CD14" *Proc. Natl. Acad. Sci. USA* 90:2744-2748 (1993).
- Rhee et al., "Studies of Inositol Phospholipid-Specific Phospholipase C" *Science* 244:546-550 (1989).
- Rosenthal et al., "Primary Structure and Biological Activity of a Novel Human Neurotrophic Factor" *Neuron* 4:767-773 (May 1990).
- Sajjadi et al., "Identification of a new eph-related receptor tyrosine kinase gene from mouse and chicken that is developmentally regulated and encodes at least two forms of the receptor" *New Biol.* 3(8):769-778 (1991).
- Sanchez et al., "Renal agenesis and the absence of enteric neurons in mice lacking GDNF" *Nature* 382:70-73 (1996).
- Sanicola et al., "Glial cell line-derived neurotrophic factor-dependent RET activation can be mediated by two different cell-surface accessory proteins" *Proc. Natl. Acad. Sci. USA* 94(12):6238-6243 (Jun. 10, 1997).
- Shukla, S., "Phosphatidylinositol Specific Phospholipases C" *Life. Sci.* 30:1323-1335 (1982).
- Stahl et al., "The Alphas, Betas, and Kinases of Cytokine Receptor Complexes" *Cell* 74:587-590 (Aug. 27, 1993).
- Stahl et al., "The Tripartite CNTF Receptor Complex: Activation and Signaling Involves Components Shared with Other Cytokines" *Journal of Neurobiology* 25(11):1454-1466 (1994).
- Stromberg et al., "Glial Cell Line-Derived Neurotrophic Factor is Expressed in the Developing but Not Adult Striatum and Stimulates Developing Dopamine Neurons in Vivo" *Exp. Neurol.* 124:401-412 (1993).
- Sun et al., "The Cystine-Knot Growth-Factor Superfamily" *Annual Review of Biophysics and Biomolecular Structure* 24:269-291 (1995).
- Takahashi et al., "Identification of the ret proto-oncogene products in neuroblastoma and leukemia cells" *Oncogene* 6:297-301 (1991).
- Thoenen et al., "Physiology of Nerve Growth Factor" *Physiological Reviews* 60(4):1284-1335 (Oct. 1980).
- Tomac et al., "Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo" *Nature* 373:335-339 (1995).
- Treanor et al., "Characterization of a multicomponent receptor for GDNF" *Nature* 382:80-83 (1996).
- Trupp et al., "Functional receptor for GDNF encoded by the c-ret proto-oncogene" *Nature* 381:785-789 (1996).
- Wrana et al., "Mechanism of activation of the TGF- β receptor" *Nature* 370:341-347 (1994).
- Yan et al., "In vivo neurotrophic effects of GDNF on the neonatal and adult facial motor neurons" *Nature* 373:341-344 (1995).

* cited by examiner


```

100 CCGAGAGCTG CGGGGGGGAGG AGGAGGAGGG TGCCGACGCT TGAGTGGTT CGAGCCCGAG CCGTAGCCGG GGGAGCCAGT CAGTTTCCGG CCAAGGCAGC
182 AGGGAGAAAG ACAAAAAAAC GGTGGGATTT ATTTAAC ATG ATC TTG GCA AAC GTC TTC CTC TTC TTC TTC TTT CTA GAC GAG
Met Ile Leu Ala Asn Val Phe Phe Leu Phe Phe Phe Phe Leu Asp Glu
1 5 10 15
257 ACC CTC CGC TCT TTG GCC AGC CCT TCC TCC CTG CAG GAC CCC GAG CTC CAC GGC TGG CGC CCC CCA GTG GAC TGT
Thr Leu Arg Ser Leu Ala Ser Pro Ser Ser Leu Gln Asp Pro Glu Leu His Gly Trp Arg Pro Pro Val Asp Cys
20 25 30 35 40
332 GTC CGG GCC AAT GAG CTG TGT GCC GAA TCC AAC TGC AGC TCT CGC TAC CGC ACT CTG CGG CAG TGC CTG GCA
Val Arg Ala Asn Glu Leu Cys Ala Ala Glu Ser Ser Asn Cys Ser Ser Arg Tyr Arg Thr Leu Arg Gln Cys Leu Ala
45 50 55 60 65
407 GGC CGC GAC CGC AAC ACC ATG GGC GGC ATG AAC GAG TGC CAG GCG GCC TTG GAG GTC TTG CAG GAG AGC CCG CTG
Gly Arg Asp Arg Asn Thr Met Leu Ala Ala Asn Lys Glu Cys Gln Ala Ala Leu Glu Val Leu Gln Glu Ser Pro Leu
70 75 80 85 90
482 TAC GAC TGC CGC TGC AAG CGG GGC ATG AAG GAG CTG CAG TGT CTG CAG ATC TAC TGG AGC ATC CAC CTG GGG
Tyr Asp Cys Arg Cys Lys Arg Gly Met Lys Lys Glu Leu Gln Cys Leu Gln Ile Tyr Trp Ser Ile His Leu Gly
95 100 105 110 115
557 CTG ACC GAG GGT GAG GAG TTC TAC GAA GCC TCC CCC TAT GAG CCG GTG ACC TCC CGC CTC TCG GAC ATC TTC AGG
Leu Thr Glu Gly Glu Glu Phe Tyr Glu Ala Ser Pro Tyr Glu Pro Val Thr Ser Arg Leu Ser Asp Ile Phe Arg
120 125 130 135 140
632 CTT GCT TCA ATC TTC TCA GGG ACA GGG GCA GAC CCG GTG GTC AGC GCC AAG AGC AAC CAT TGC CTG GAT GCT GCC
Leu Ala Ser Ile Phe Ser Gly Thr Gly Ala Asp Pro Val Ser Ala Lys Ser Asn His Cys Leu Asp Ala Ala
145 150 155 160 165
707 AAG GCC TGC AAC CTG AAT GAC AAC TGC AAC GAG TGC AAC GGC TCC TCC TAC ATC TGC AAC CGC GAG ATC TCG
Lys Ala Cys Asn Leu Asn Asp Asn Cys Lys Leu Arg Ser Ser Tyr Ile Ser Ile Cys Asn Arg Glu Ile Ser
170 175 180 185 190
782 CCC ACC GAG CGC TGC AAC CGC CGC AAG TGC CAC AAG GCC CTG CGC CAG TTC TTC GAC CGG GTG CCC AGC GAG TAC
Pro Thr Glu Arg Cys Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Asp Arg Val Pro Ser Glu Tyr
195 200 205 210 215

```

FIG. 1A

ACC TAC CGC ATG CTC TTC TGC TCC TGC CAA GAC CAG GCG TGC GCT GAG CGC CGC CGG CAA ACC ATC CTG CCC AGC 857
 Thr Tyr Arg Met Leu Phe Cys Ser Cys Gln Asp Gln Ala Cys Ala Glu Arg Arg Arg Gln Thr Ile Leu Pro Ser 240
 220 225
 TGC TCC TAT GAG GAC AAG GAG AAG CCC AAC TGC CTG GAC CTG CGT CGT GGC GTG TGC CGG ACT GAC CAC CTG TGT CGG 932
 Cys Ser Tyr Glu Asp Lys Glu Lys Pro Asn Cys Leu Asp Leu Arg Gly Val Cys Arg Thr Asp His Leu Cys Arg 265
 245 250 255 260
 TCC CGG CTG GCC GAC TTC CAT GCC AAT TGT CGA GCC TCC TAC CAG ACG GTC ACC AGC TGC CCT GCG GAC AAT TAC 1007
 Ser Arg Leu Ala Asp Phe His Ala Asn Cys Arg Ala Ser Tyr Gln Thr Val Thr Ser Cys Pro Ala Asp Asn Tyr 290
 270 275 280 285
 CAG GCG TGT CTG GGC TCT TAT GCT GGC ATG ATT GGG TTT GAC ATG ACA CCT AAC TAT GTG GAC TCC AGC CCC ACT 1082
 Gln Ala Cys Leu Gly Ser Tyr Ala Ala Gly Met Ile Gly Phe Asp Met Thr Pro Asn Tyr Val Asp Ser Ser Pro Thr 315
 295 300 305 310
 GGC ATC GTG TCC CCC TGG TGC AGC TGT CGT GGC AGC GGG AAC ATG GAG GAG GAG TGT GAG AAG TTC CTC AGG 1157
 Gly Ile Val Val Ser Pro Trp Cys Ser Cys Arg Gly Ser Gly Asn Met Glu Glu Cys Glu Lys Phe Leu Arg 340
 320 325 330 335
 GAC TTC ACC GAG AAC CCA TGC CTC CGG AAC GCC ATC CAG GCC TTT GGC AAC GGC ACG GAC GTG AAC GTG TCC CCA 1232
 Asp Phe Thr Glu Asn Pro Cys Leu Arg Asn Ala Ile Gln Ala Phe Gly Asn Gly Thr Asp Val Asn Val Ser Pro 365
 345 350 355 360
 AAA GGC CCC TCG TTC CAG GCC ACC CAG GCC CCT CGG GTG GAG AAG ACG CCT TCT TTG CCA GAT GAC CTC AGT GAC 1307
 Lys Gly Pro Ser Phe Gln Ala Thr Gln Ala Pro Arg Val Glu Lys Thr Pro Ser Leu Pro Asp Asp Leu Ser Asp 390
 370 375 380 385
 AGT ACC AGC TTG GGG ACC AGT GTC ATC ACC ACC TGC ACG TCT GTC CAG GAG CAG GGG CTG AAG GCC AAC AAC TCC 1382
 Ser Thr Ser Leu Gly Thr Ser Val Ile Thr Thr Cys Thr Ser Val Gln Glu Gln Gly Leu Lys Ala Asn Asn Ser 415
 395 400 405 410 415
 AAA GAG TTA AGC ATG TGC TTC ACA GAG CTC ACG ACA AAT ATC ATC CCA GGG AGT AAC AAG GTG ATC AAA CCT AAC 1457
 Lys Glu Leu Ser Met Cys Phe Thr Glu Leu Thr Thr Asn Ile Ile Pro Gly Ser Asn Lys Val Ile Lys Pro Asn 440
 420 425 430 435 440

FIG. 1B

TCA GGC CCC AGC AGA GCC AGA CCG TCG GCT GCC TTG ACC GTG CTG TCT GTC CTG ATG CTG AAA CTG GCC TTG T 1530
 Ser Gly Pro Ser Arg Ala Arg Pro Ser Ala Ala Leu Thr Val Leu Ser Val Leu Met Leu Lys Leu Ala Leu (SEQIDNO:3)
 445 450 455 460 464
 AGGCTGTGGG AACCGAGTCA GAATATTTT GAAAGCTACG CAGACAAGAA CAGCCGCCCTG ACGAAATGGA AACACACACA GACACACACA CACCTTGCAA 1630
 AAAAAAATT GTTTTCCCA CCTGTGCGCT GAACCTGTCT CCTCCCAGGT TTCTTCTCTG GAGAAGTTTT TGTAACCCAA ACAGACAAGC AGGCAGGCAG 1730
 CCTGAGAGCT GGCCCAAGGG TCCCCTGGCA GGGGAAACTC TGGTGCCGGG GAGGCCACGA GGCTCTAGAA ATGCCCTTCA CTTTCTCCTG GTGTTTTTCT 1830
 CTCTGGACCC TTCTGAAGCA GAGACCGGAC AAGAGCCTGC AGCGGAAGG ACTCTGGGCT GTGCCCTGAGG CTGGCTGGGG GCAGGACAAC ACAGCTGCTT 1930
 CCCCAGGCTG CCCACTCTGG GGACCCCGCTG GGGGCTGGCA GAGGGCATCG GTCAGCCGGG CAGCCGGGCT GGCCATGAGG GTCCACCTTC AGCCCTTTGG 2030
 CTTCAAGGAT GGAGATGGTT TTGCCCTCCC TCCTGCCCCT CCGGTGGGGC TGGTGGGTCT GCAGCTGGTG TGGGAACCTC CCCACGGATG GCGGTGGAGG 2130
 GGGTTCCAC CGTGCTGGC TCCCCCTGAC TGTAGCACCG AGTGTGGGG CTGGGGGCCA GCTCCAGGAG GGCTTGAGAG CTCAGCCTGC CTGGGAGAGC 2230
 CCTGTGGCG AGGCATATAA ACTTGGGCAC CAGCTTCTTT CTCGGTGGCA GAAATTTGA AGTCAGAGAG AAACGGTCTT TTGTTGGCTT CTTTGTCTTC 2330
 TCGTGGGTCC TTTGGCAGGC CTCCTTTGG CTTCCCTTTGG GGAGAGACC ACAGCCGGGT GTGTGTCTGC AGCACCGTGG GCCCTCAAGC TTTCCCTGCTG 2430
 TCTTCTCCCT CCTCCTCCTT TCCCCTTCT CTTTCTCTCAT TTCTAGAGG TACGTCAACT GTATGTACAT ACCGGGGCTC CTCTCCTAAC ATATATGTAT 2530
 ATACACATCC ATATACATAT ATTGTGTGGT TTCCCCTTTC TTTCCCTTTT TAAGCAACA AACTATGGGG(SEQIDNO:1) 2600

FIG. 1C

100 GCGGTGGCGG CCGCTCTAGA ACTAGTGGAT CCCCAGGCTG CAGGAATTCG GCACGAGAGT GAGCCGAGCA AGGGTTAGCG GGAGAAGATT TTTTTTTTTT
 200 TGAATCTTTT TCTTCGTCTT GGTGCCAAG AAGGACTCT GGTCTCCCGT CCTAGAAGCT CCTACTGGAT TGCTCCTATT CCGTCGGTGG ATTTCTTTCC
 300 TATTCCGATT TATTCTGACC CCGTCCCTCG CTGCTTCCCTT CCAGCCCTTC ACTTTCAGAT CGCCTCGCCC CCACCTCTCC AGTCCCCTCC TGGGAAGTGC
 400 AGGGAAITG GACCCACGGG GACTCAGCC TTCCCGGACG CCGGAGCAA GGGCTGGGCT GACCTCAGGA CCAGGCTGTT GGCTTAGAAG GCAGCCAGAC
 500 ACATAGCTAC GTGTGTTGA TTTCAGTGGC AAGGGGGAC GTCGAGAGGC AGCCACCCG CCGCTCCTA CCCCCTCCCC TCCAACCAGC AGTGAGAATC
 600 CCAGGACTCG GGATCTTCAA CCGGCGGCGG CCGGCGGCGG TCTCCGCATT GGATTTGGG GTCGTTATTG CTCGGCTGTT ATTATTATCG TTATTTTATT
 688 TTTATTTTTT AAACCTAAGG GAGAAAGACA CATAACACA AACTGTGGG ATTTATTAA C ATG ATC TTG GCA AAC GCC TTC TGC CTC TTC
 Met Ile Leu Ala Asn Ala Phe Cys Leu Phe
 1 5 10
 15 20 25 30 35
 40 45 50 55 60
 65 70 75 80 85
 90 95 100 105 110
 115 120 125 130 135
 135 140 145 150 155
 160 165 170 175 180
 185 190 195 200 205
 210 215 220 225 230
 235 240 245 250 255
 260 265 270 275 280
 285 290 295 300 305
 310 315 320 325 330
 335 340 345 350 355
 360 365 370 375 380
 385 390 395 400 405
 410 415 420 425 430
 435 440 445 450 455
 460 465 470 475 480
 485 490 495 500 505
 510 515 520 525 530
 535 540 545 550 555
 560 565 570 575 580
 585 590 595 600 605
 610 615 620 625 630
 635 640 645 650 655
 660 665 670 675 680
 685 690 695 700 705
 710 715 720 725 730
 735 740 745 750 755
 760 765 770 775 780
 785 790 795 800 805
 810 815 820 825 830
 835 840 845 850 855
 860 865 870 875 880
 885 890 895 900 905
 910 915 920 925 930
 935 940 945 950 955
 960 965 970 975 980
 985 990 995 1000 1005
 1010 1015 1020 1025 1030
 1035 1040 1045 1050 1055
 1060 1065 1070 1075 1080
 1085 1090 1095 1100 1105
 1110 1115 1120 1125 1130
 1135 1140 1145 1150 1155
 1160 1165 1170 1175 1180
 1185 1190 1195 1200 1205
 1210 1215 1220 1225 1230
 1235 1240 1245 1250 1255
 1260 1265 1270 1275 1280
 1285 1290 1295 1300 1305
 1310 1315 1320 1325 1330
 1335 1340 1345 1350 1355
 1360 1365 1370 1375 1380
 1385 1390 1395 1400 1405
 1410 1415 1420 1425 1430
 1435 1440 1445 1450 1455
 1460 1465 1470 1475 1480
 1485 1490 1495 1500 1505
 1510 1515 1520 1525 1530
 1535 1540 1545 1550 1555
 1560 1565 1570 1575 1580
 1585 1590 1595 1600 1605
 1610 1615 1620 1625 1630
 1635 1640 1645 1650 1655
 1660 1665 1670 1675 1680
 1685 1690 1695 1700 1705
 1710 1715 1720 1725 1730
 1735 1740 1745 1750 1755
 1760 1765 1770 1775 1780
 1785 1790 1795 1800 1805
 1810 1815 1820 1825 1830
 1835 1840 1845 1850 1855
 1860 1865 1870 1875 1880
 1885 1890 1895 1900 1905
 1910 1915 1920 1925 1930
 1935 1940 1945 1950 1955
 1960 1965 1970 1975 1980
 1985 1990 1995 2000 2005
 2010 2015 2020 2025 2030
 2035 2040 2045 2050 2055
 2060 2065 2070 2075 2080
 2085 2090 2095 2100 2105
 2110 2115 2120 2125 2130
 2135 2140 2145 2150 2155
 2160 2165 2170 2175 2180
 2185 2190 2195 2200 2205
 2210 2215 2220 2225 2230
 2235 2240 2245 2250 2255
 2260 2265 2270 2275 2280
 2285 2290 2295 2300 2305
 2310 2315 2320 2325 2330
 2335 2340 2345 2350 2355
 2360 2365 2370 2375 2380
 2385 2390 2395 2400 2405
 2410 2415 2420 2425 2430
 2435 2440 2445 2450 2455
 2460 2465 2470 2475 2480
 2485 2490 2495 2500 2505
 2510 2515 2520 2525 2530
 2535 2540 2545 2550 2555
 2560 2565 2570 2575 2580
 2585 2590 2595 2600 2605
 2610 2615 2620 2625 2630
 2635 2640 2645 2650 2655
 2660 2665 2670 2675 2680
 2685 2690 2695 2700 2705
 2710 2715 2720 2725 2730
 2735 2740 2745 2750 2755
 2760 2765 2770 2775 2780
 2785 2790 2795 2800 2805
 2810 2815 2820 2825 2830
 2835 2840 2845 2850 2855
 2860 2865 2870 2875 2880
 2885 2890 2895 2900 2905
 2910 2915 2920 2925 2930
 2935 2940 2945 2950 2955
 2960 2965 2970 2975 2980
 2985 2990 2995 3000 3005
 3010 3015 3020 3025 3030
 3035 3040 3045 3050 3055
 3060 3065 3070 3075 3080
 3085 3090 3095 3100 3105
 3110 3115 3120 3125 3130
 3135 3140 3145 3150 3155
 3160 3165 3170 3175 3180
 3185 3190 3195 3200 3205
 3210 3215 3220 3225 3230
 3235 3240 3245 3250 3255
 3260 3265 3270 3275 3280
 3285 3290 3295 3300 3305
 3310 3315 3320 3325 3330
 3335 3340 3345 3350 3355
 3360 3365 3370 3375 3380
 3385 3390 3395 3400 3405
 3410 3415 3420 3425 3430
 3435 3440 3445 3450 3455
 3460 3465 3470 3475 3480
 3485 3490 3495 3500 3505
 3510 3515 3520 3525 3530
 3535 3540 3545 3550 3555
 3560 3565 3570 3575 3580
 3585 3590 3595 3600 3605
 3610 3615 3620 3625 3630
 3635 3640 3645 3650 3655
 3660 3665 3670 3675 3680
 3685 3690 3695 3700 3705
 3710 3715 3720 3725 3730
 3735 3740 3745 3750 3755
 3760 3765 3770 3775 3780
 3785 3790 3795 3800 3805
 3810 3815 3820 3825 3830
 3835 3840 3845 3850 3855
 3860 3865 3870 3875 3880
 3885 3890 3895 3900 3905
 3910 3915 3920 3925 3930
 3935 3940 3945 3950 3955
 3960 3965 3970 3975 3980
 3985 3990 3995 4000 4005
 4010 4015 4020 4025 4030
 4035 4040 4045 4050 4055
 4060 4065 4070 4075 4080
 4085 4090 4095 4100 4105
 4110 4115 4120 4125 4130
 4135 4140 4145 4150 4155
 4160 4165 4170 4175 4180
 4185 4190 4195 4200 4205
 4210 4215 4220 4225 4230
 4235 4240 4245 4250 4255
 4260 4265 4270 4275 4280
 4285 4290 4295 4300 4305
 4310 4315 4320 4325 4330
 4335 4340 4345 4350 4355
 4360 4365 4370 4375 4380
 4385 4390 4395 4400 4405
 4410 4415 4420 4425 4430
 4435 4440 4445 4450 4455
 4460 4465 4470 4475 4480
 4485 4490 4495 4500 4505
 4510 4515 4520 4525 4530
 4535 4540 4545 4550 4555
 4560 4565 4570 4575 4580
 4585 4590 4595 4600 4605
 4610 4615 4620 4625 4630
 4635 4640 4645 4650 4655
 4660 4665 4670 4675 4680
 4685 4690 4695 4700 4705
 4710 4715 4720 4725 4730
 4735 4740 4745 4750 4755
 4760 4765 4770 4775 4780
 4785 4790 4795 4800 4805
 4810 4815 4820 4825 4830
 4835 4840 4845 4850 4855
 4860 4865 4870 4875 4880
 4885 4890 4895 4900 4905
 4910 4915 4920 4925 4930
 4935 4940 4945 4950 4955
 4960 4965 4970 4975 4980
 4985 4990 4995 5000 5005
 5010 5015 5020 5025 5030
 5035 5040 5045 5050 5055
 5060 5065 5070 5075 5080
 5085 5090 5095 5100 5105
 5110 5115 5120 5125 5130
 5135 5140 5145 5150 5155
 5160 5165 5170 5175 5180
 5185 5190 5195 5200 5205
 5210 5215 5220 5225 5230
 5235 5240 5245 5250 5255
 5260 5265 5270 5275 5280
 5285 5290 5295 5300 5305
 5310 5315 5320 5325 5330
 5335 5340 5345 5350 5355
 5360 5365 5370 5375 5380
 5385 5390 5395 5400 5405
 5410 5415 5420 5425 5430
 5435 5440 5445 5450 5455
 5460 5465 5470 5475 5480
 5485 5490 5495 5500 5505
 5510 5515 5520 5525 5530
 5535 5540 5545 5550 5555
 5560 5565 5570 5575 5580
 5585 5590 5595 5600 5605
 5610 5615 5620 5625 5630
 5635 5640 5645 5650 5655
 5660 5665 5670 5675 5680
 5685 5690 5695 5700 5705
 5710 5715 5720 5725 5730
 5735 5740 5745 5750 5755
 5760 5765 5770 5775 5780
 5785 5790 5795 5800 5805
 5810 5815 5820 5825 5830
 5835 5840 5845 5850 5855
 5860 5865 5870 5875 5880
 5885 5890 5895 5900 5905
 5910 5915 5920 5925 5930
 5935 5940 5945 5950 5955
 5960 5965 5970 5975 5980
 5985 5990 5995 6000 6005
 6010 6015 6020 6025 6030
 6035 6040 6045 6050 6055
 6060 6065 6070 6075 6080
 6085 6090 6095 6100 6105
 6110 6115 6120 6125 6130
 6135 6140 6145 6150 6155
 6160 6165 6170 6175 6180
 6185 6190 6195 6200 6205
 6210 6215 6220 6225 6230
 6235 6240 6245 6250 6255
 6260 6265 6270 6275 6280
 6285 6290 6295 6300 6305
 6310 6315 6320 6325 6330
 6335 6340 6345 6350 6355
 6360 6365 6370 6375 6380
 6385 6390 6395 6400 6405
 6410 6415 6420 6425 6430
 6435 6440 6445 6450 6455
 6460 6465 6470 6475 6480
 6485 6490 6495 6500 6505
 6510 6515 6520 6525 6530
 6535 6540 6545 6550 6555
 6560 6565 6570 6575 6580
 6585 6590 6595 6600 6605
 6610 6615 6620 6625 6630
 6635 6640 6645 6650 6655
 6660 6665 6670 6675 6680
 6685 6690 6695 6700 6705
 6710 6715 6720 6725 6730
 6735 6740 6745 6750 6755
 6760 6765 6770 6775 6780
 6785 6790 6795 6800 6805
 6810 6815 6820 6825 6830
 6835 6840 6845 6850 6855
 6860 6865 6870 6875 6880
 6885 6890 6895 6900 6905
 6910 6915 6920 6925 6930
 6935 6940 6945 6950 6955
 6960 6965 6970 6975 6980
 6985 6990 6995 7000 7005
 7010 7015 7020 7025 7030
 7035 7040 7045 7050 7055
 7060 7065 7070 7075 7080
 7085 7090 7095 7100 7105
 7110 7115 7120 7125 7130
 7135 7140 7145 7150 7155
 7160 7165 7170 7175 7180
 7185 7190 7195 7200 7205
 7210 7215 7220 7225 7230
 7235 7240 7245 7250 7255
 7260 7265 7270 7275 7280
 7285 7290 7295 7300 7305
 7310 7315 7320 7325 7330
 7335 7340 7345 7350 7355
 7360 7365 7370 7375 7380
 7385 7390 7395 7400 7405
 7410 7415 7420 7425 7430
 7435 7440 7445 7450 7455
 7460 7465 7470 7475 7480
 7485 7490 7495 7500 7505
 7510 7515 7520 7525 7530
 7535 7540 7545 7550 7555
 7560 7565 7570 7575 7580
 7585 7590 7595 7600 7605
 7610 7615 7620 7625 7630
 7635 7640 7645 7650 7655
 7660 7665 7670 7675 7680
 7685 7690 7695 7700 7705
 7710 7715 7720 7725 7730
 7735 7740 7745 7750 7755
 7760 7765 7770 7775 7780
 7785 7790 7795 7800 7805
 7810 7815 7820 7825 7830
 7835 7840 7845 7850 7855
 7860 7865 7870 7875 7880
 7885 7890 7895 7900 7905
 7910 7915 7920 7925 7930
 7935 7940 7945 7950 7955
 7960 7965 7970 7975 7980
 7985 7990 7995 8000 8005
 8010 8015 8020 8025 8030
 8035 8040 8045 8050 8055
 8060 8065 8070 8075 8080
 8085 8090 8095 8100 8105
 8110 8115 8120 8125 8130
 8135 8140 8145 8150 8155
 8160 8165 8170 8175 8180
 8185 8190 8195 8200 8205
 8210 8215 8220 8225 8230
 8235 8240 8245 8250 8255
 8260 8265 8270 8275 8280
 8285 8290 8295 8300 8305
 8310 8315 8320 8325 8330
 8335 8340 8345 8350 8355
 8360 8365 8370 8375 8380
 8385 8390 8395 8400 8405
 8410 8415 8420 8425 8430
 8435 8440 8445 8450 8455
 8460 8465 8470 8475 8480
 8485 8490 8495 8500 8505
 8510 8515 8520 8525 8530
 8535 8540 8545 8550 8555
 8560 8565 8570 8575 8580
 8585 8590 8595 8600 8605
 8610 8615 8620 8625 8630
 8635 8640 8645 8650 8655
 8660 8665 8670 8675 8680
 8685 8690 8695 8700 8705
 8710 8715 8720 8725 8730
 8735 8740 8745 8750 8755
 8760 8765 8770 8775 8780
 8785 8790 8795 8800 8805
 8810 8815 8820 8825 8830
 8835 8840 8845 8850 8855
 8860 8865 8870 8875 8880
 8885 8890 8895 8900 8905
 8910 8915 8920 8925 8930
 8935 8940 8945 8950 8955
 8960 8965 8970 8975 8980
 8985 8990 8995 9000 9005
 9010 9015 9020 9025 9030
 9035 9040 9045 9050 9055
 9060 9065 9070 9075 9080
 9085 9090 9095 9100 9105
 9110 9115 9120 9125 9130
 9135 9140 9145 9150 9155
 9160 9165 9170 9175 9180
 9185 9190 9195 9200 9205
 9210 9215 9220 9225 9230
 9235 9240 9245 9250 9255
 9260 9265 9270 9275 9280
 9285 9290 9295 9300 9305
 9310 9315 9320 9325 9330
 9335 9340 9345 9350 9355
 9360 9365 9370 9375 9380
 9385 9390 9395 9400 9405
 9410 9415 9420 9425 9430
 9435 9440 9445 9450 9455
 9460 9465 9470 9475 9480
 9485 9490 9495 9500 9505
 9510 9515 9520 9525 9530
 9535 9540 9545 9550 9555
 9560 9565 9570 9575 9580
 9585 9590 9595 9600 9605
 9610 9615 9620 9625 9630
 9635 9640 9645 9650 9655
 9660 9665 9670 9675 9680
 9685 9690 9695 9700 9705
 9710 9715 9720 9725 9730
 9735 9740 9745 9750 9755
 9760 9765 9770 9775 9780
 9785 9790 9795 9800 9805
 9810 9815 9820 9825 9830
 9835 9840 9845 9850 9855
 9860 9865 9870 9875 9880
 9885 9890 9895 9900 9905
 9910 9915 9920 9925 9930
 9935 9940 9945 9950 9955
 9960 9965 9970 9975 9980
 9985 9990 9995 10000 10005
 10010 10015 10020 10025 10030
 10035 10040 10045 10050 10055
 10060 10065 10070 10075 10080
 10085 10090 10095 10100 10105
 10110 10115 10120 10125 10130
 10135 10140 10145 10150 10155
 10160 10165 10170 10175 10180
 10185 10190 10195 10200 10205
 10210 10215 10220 10225 10230
 10235 10240 10245 10250 10255

1141 TCG GAC ATC TTC AGG CTC GCT TCA ATC TTC TCA GGG ACA GGG ACA GAC CCG GCG GTC AGT ACC AAA AGC AAC CAC
 Ser Asp Ile Phe Arg Leu Ala Ser Ile Phe Ser Gly Thr Gly Thr Asp Pro Ala Val Ser Thr Lys Ser Asn His 160
 140 145 150 155
 1216 TGC CTG GAT GCC AAG GCC TGC AAC CTG AAT GAC AAC TGC AAG AAG CTT CGC TCC TCT TAT ATC TCC ATC TGC
 Cys Leu Asp Ala Ala Lys Ala Cys Asn Leu Asn Asp Asn Cys Lys Lys Leu Arg Ser Ser Tyr Ile Ser Ile Cys 185
 165 170 175 180
 1291 AAC CGT GAG ATC TCT CCC ACC GAA CGC TGC AAC CGC CGC AAG GCT CTG CGC CAG TTC TTT GAC CGT
 Asn Arg Glu Ile Ser Pro Thr Glu Arg Cys Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Phe Asp Arg 210
 190 195 200 205
 1366 GTG CCC AGC GAG TAT ACC TAC CGC ATG CTC TTC TGC TCC TGT TGC CAG GAC GCA TGT GCT GAG CGT CGC CGG CAA
 Val Pro Ser Glu Tyr Thr Tyr Arg Met Leu Phe Cys Ser Cys Gln Asp Gln Ala Cys Ala Glu Arg Arg Gln 235
 215 220 225 230
 1441 ACC ATC CTG CCC AGT TGC TCC TAT GAG GAC AAG GAG AAG CCC AAC TGC CTG GAC CTG CGC AGC CTG TGT CGT ACA
 Thr Ile Leu Pro Ser Cys Ser Tyr Glu Asp Lys Glu Lys Pro Asn Cys Leu Asp Leu Arg Ser Leu Cys Arg Thr 260
 240 245 250 255
 1516 GAC CAC CTG TGC CGG TCC CGA CTG GCA GAT TTC CAC GCC AAC TGT CGA GCC TCC TAC CGG ACA ATC ACC AGC TGT
 Asp His Leu Cys Arg Ser Arg Leu Ala Asp Phe His Ala Asn Cys Arg Ala Ser Tyr Arg Thr Ile Thr Ser Cys 285
 265 270 275 280
 1591 CCT GCG GAC AAC TAC CAG GCA TGT CTG GGC TCC TAT GCT GGC ATG ATT GGG TTT GAT ATG ACA CCC AAC TAT GTG
 Pro Ala Asp Asn Tyr Gln Ala Cys Leu Gly Ser Tyr Ala Gly Met Ile Gly phe Asp Met Thr Pro Asn Tyr Val 310
 290 295 300 305

FIG. 2B

GAC TCC AAC CCC ACG GGC ATC GTG GTG TCT CCC TGG TGC AAT TGT CGT GGC AGT GGG AAC ATG GAA GAA GAG TGT	1666
Asp Ser Asn Pro Thr Gly Ile Val Val Ser Pro Trp Cys Asn Cys Arg Gly Ser Gly Asn Met Glu Glu Cys	335
315	330
320	325
GAG AAG TTC CTC AGG GAC TTC ACG GAA AAC CCA TGC CTC CGG AAT GCC ATT CAG GCC TTT GGT AAT GGC ACA GAT	1741
Glu Lys Phe Leu Arg Asp Phe Thr Glu Asn Pro Cys Leu Arg Asn Ala Ile Gln Ala Phe Gly Asn Gly Thr Asp	360
340	355
345	350
GTG AAC ATG TCT CCC AAA GGC CCC TCA CTC CCA GCT ACC CAG GCC CCT CGG GTG GAG AAG ACT CCT TCA CTG CCA	1816
Val Asn Met Ser Pro Lys Gly Pro Ser Leu Pro Ala Thr Gln Ala Pro Arg Val Glu Lys Thr Pro Ser Leu Pro	385
365	375
370	380
GAT GAC CTC AGT GAC AGC ACC AGC CTG GGG ACC AGT GTC ATC ACC ACC TGC ACA TCT ATC CAG GAG CAA GGG CTG	1891
Asp Asp Leu Ser Asp Ser Thr Ser Leu Gly Thr Ser Val Ile Thr Thr Cys Thr Ser Ile Gln Glu Gln Gly Leu	410
390	400
395	405
AAG GCC AAC AAC TCC AAA GAG TTA AGC ATG TGC TTC ACA GAG CTC ACG ACA AAC ATC AGT CCA GGG AGT AAA AAG	1966
Lys Ala Asn Asn Ser Lys Glu Ser Met Cys Phe Thr Glu Leu Thr Thr Asn Ile Ser Pro Gly Ser Lys Lys	435
415	425
420	430
GTG ATC AAA CTT AAC TCA GGC TCC AGC AGA GCC AGA CTG TCG GCT GCC TTG ACT GCC CTC CCA CTC CTG ATG CTG	2041
Val Ile Lys Leu Asn Ser Gly Ser Ser Arg Ala Arg Leu Ser Ala Ala Leu Thr Ala Leu Pro Leu Leu Met Leu	460
440	450
445	455

FIG. 2C

ACC TTG GCC TTG T AGGCCT TTGGAACCCA GCACAAAAGT TCTTCAAGCA ACCCAGATAT GAACTCCCGC CTGACAAAAT GGAAACACAC GCATACACAC 2140

Thr Leu Ala Leu (SEQIDNO:6)

464

ATGCACACAC ACACAAACAC ACACACACAC ACACACACAC ACACACACAC ACACCCCTTG CAAAAACACT TTTTTTCCTA CATTGTCTCT 2240

GAACTTTCT CCTCCCAAGT TTCTTCTCTG GAGAAGTTT TCTAAACCAA ACAGACAAGC AGCGGGCAG TCAGAAGCCT GCCCAGAGGT CCCCTGCAAG 2340

GGACACCCAG CACCAACGAG GGCTCAAGC TCTTGAGAGA CTCTTTTCTC TTCACTGGTG TTTTCTCTCT GGACAAGATG AGACCCCTGAT GTGGAAGGTA 2440

CTTTGCTGTG CCTGGTGTGG ACTGGGGAAA GGACAGTTGC AGCTGCCCTAC TCTGGGACC TGCCCAAGG TTCACAGAGA GTCTCAGTCA GCAAGGAAGC 2540

AGGGCTGGCC ACAAGGACTT TGTACCTCT TCCCTTTGGC TTCAGAGATG GAAATGGTT GCTGCCATCC CCAGCCATTA TGTGGCCCTAG TGGGTTTAAAG 2640

TCTGGAGTAG GAAGCCTCAT GGCAGCTTCA GGCCATGGTG CCTGTAGTAT AGCTGGGGTT GGGAGCTGTT ACAGGAGGAA CTTTCTTTGG GGCATGAGCA 2740

AGCCTTGGTT GGGCACCAGC TCCAAGATGT ACCTTCCCTCC TTTATGCCAG GAATCTTGAA GTCAAAAGAGA AATGATCCTC TGTGGCTCT TTTTTGTTTG 2840

TTTTTGAATT TTTTTGTGGG TCCATTGGC AGGTCCTCT TGGGAGAAG GGCTGTTGAG TGGGGCTGGG GAGACCCTAG CTGGGCGTGT GTATGGAGCA 2940

CTCTGGTGGG TTCCCAAGCT TGCCCTTCT CTCTCTTGT TCTGCTTTC TCTCTCATTT CTGAGACATT CATGCACCTGT CTGTACATAC TGGGTCTCCT 3040

TTCTCAACAT ATGTGTATAT CCATATCCAT ATATCCTATG ATTTTACTCT TTCTTTTCAAT TTTTTTAAAG AAACAATAA TACCCTACAG 3140

ATGAGCGAAA ATGTATTATT GTAAAGTTTA TTTTTTTTAA TAATGTTGTC TATGATGGGA AGAAAGGTAC CAGGACCCCT GAGCCCTGGT CCAGTTGGGC 3240

TGGTGGGCT GTGGCCGGG ACTCCCGATT GCATTCACTC TTAACCAAGC TCCAATAAAC GTACTAGGAA GCGAAAAAAA AAAAAAAA ACTCGAGGGG 3340

GGCCCCGGTA CCAATTC (SEQIDNO:4) 3358

FIG. 2D

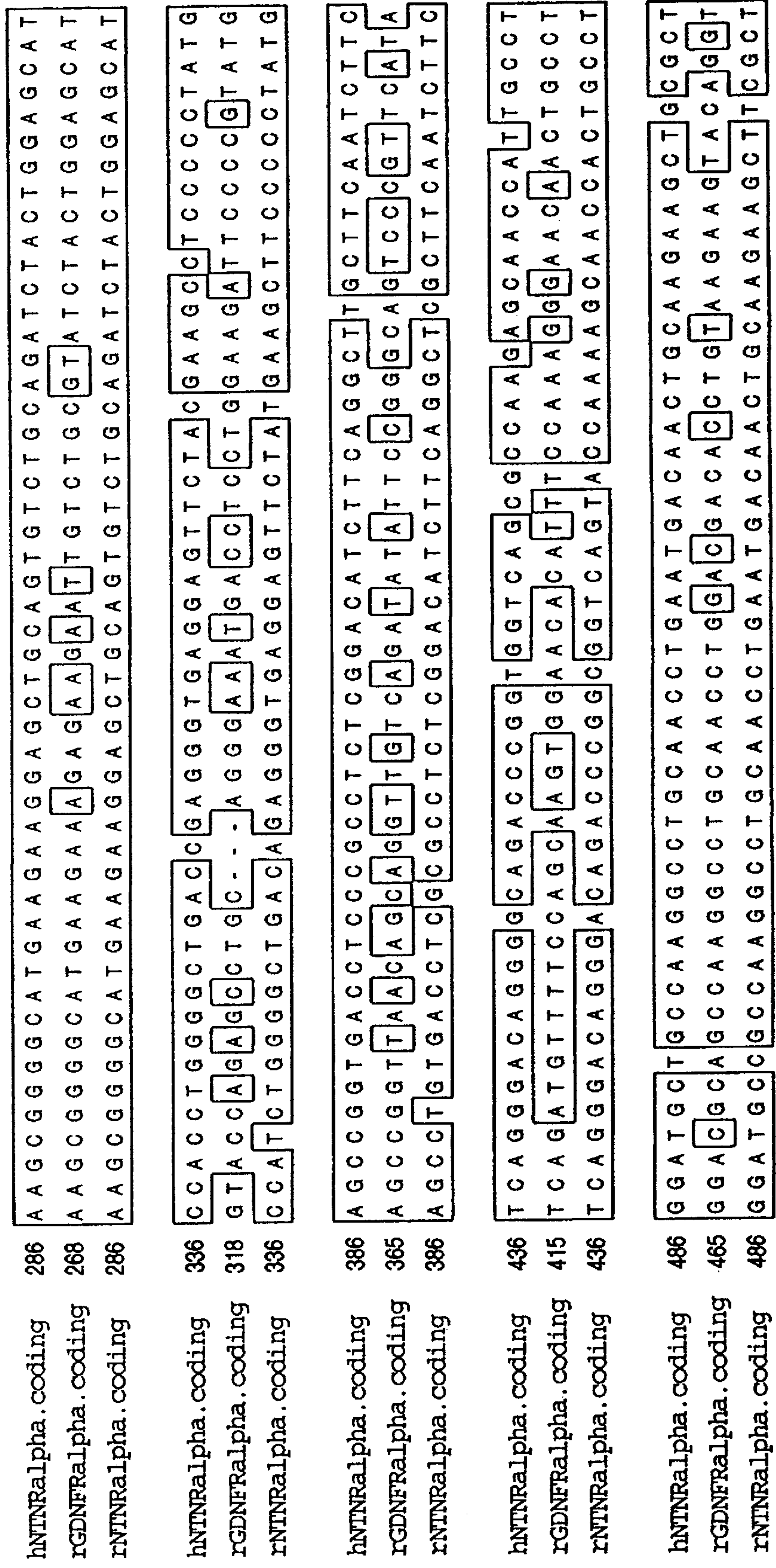


FIG. 3B

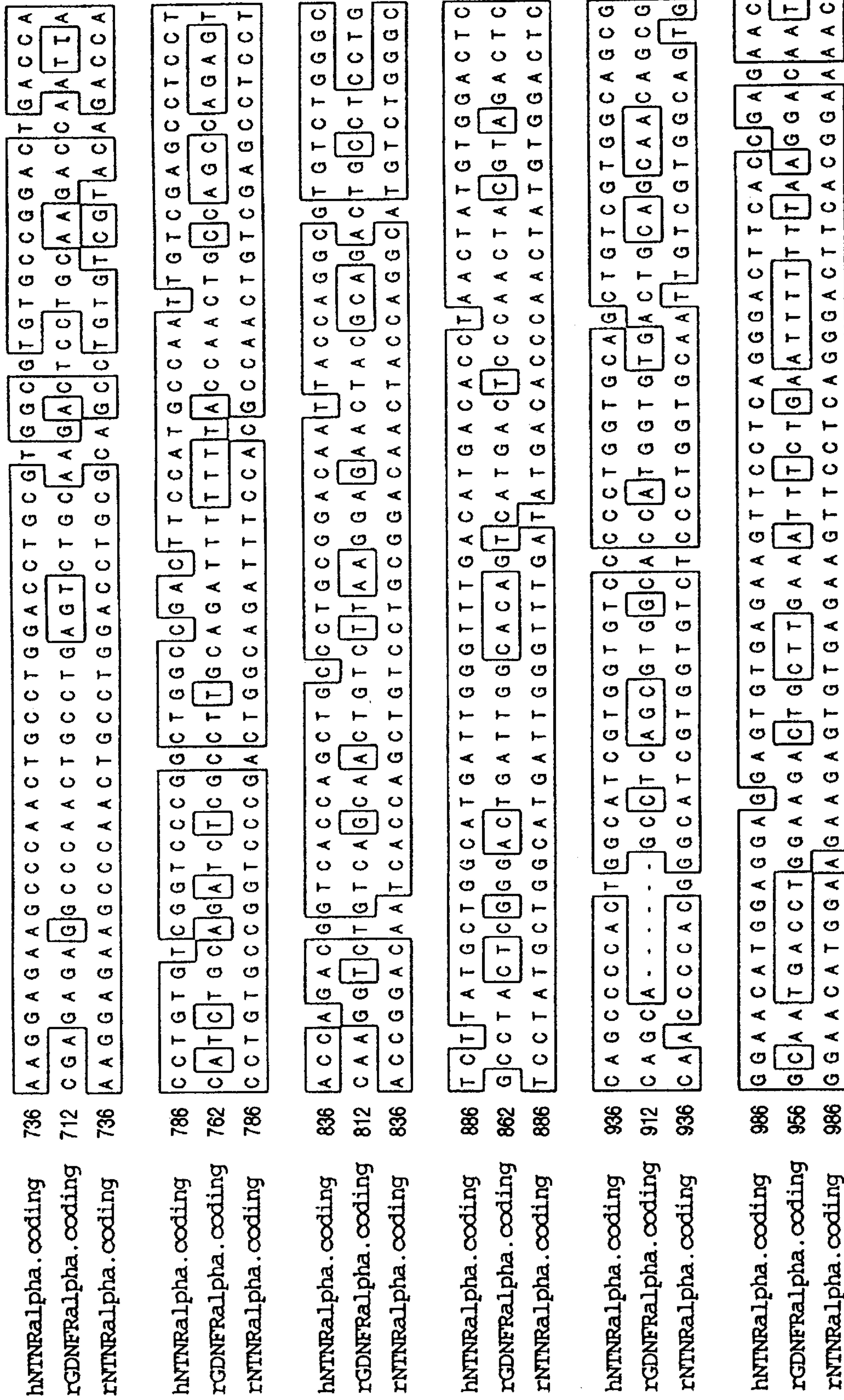


FIG. 3D

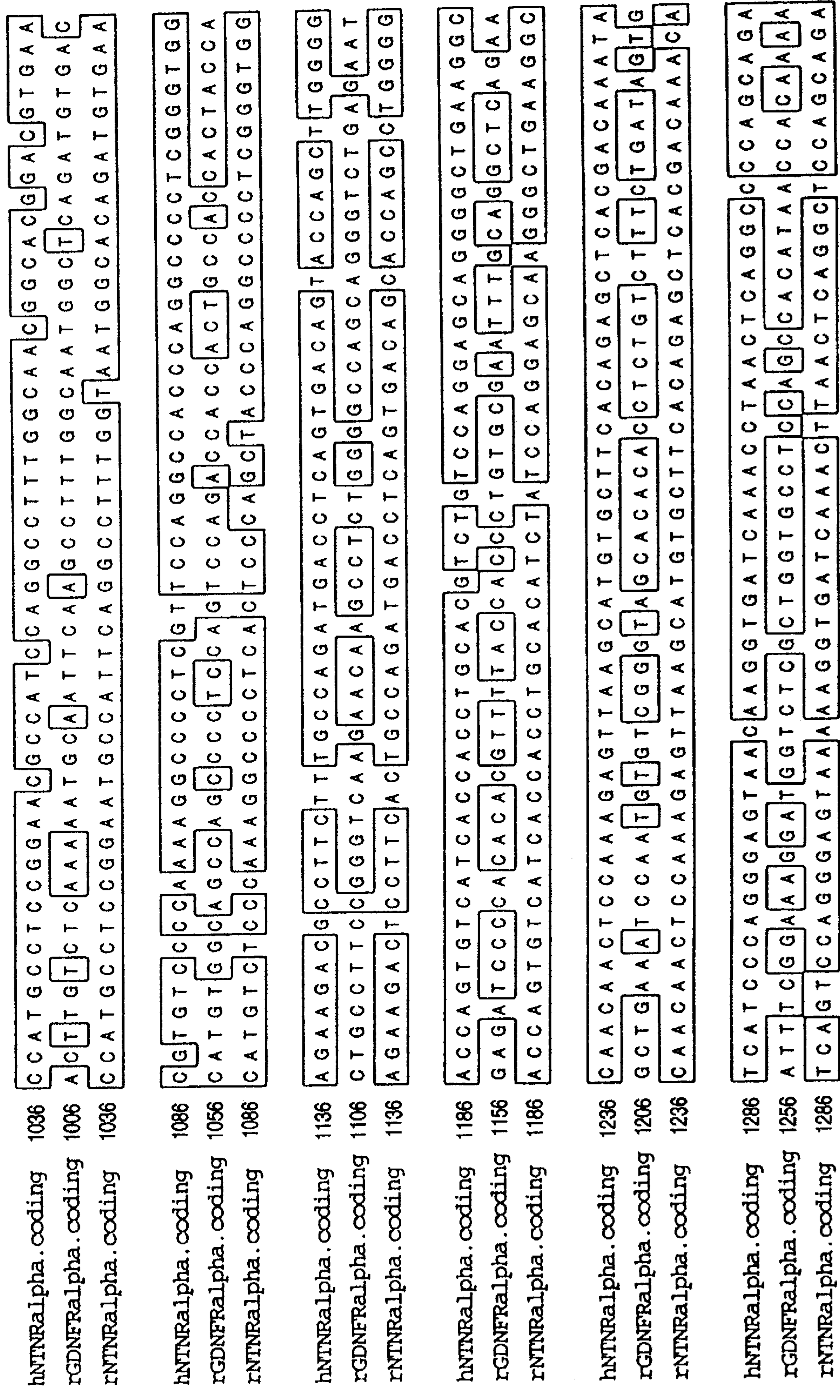


FIG. 3E

hNTNRalpha.coding 1336 G C C A G A C C G T C G G C T G C C T T G A C C G T G C T G T C T G T C C T G A T G C T G A A A C T
 rGDNFRalpha.coding 1306 T C A A T G G C T G C T C C T C C C A G C T G C A G T C T G A G C T C A C T G C C G T G C T G A A T
 rNTNRalpha.coding 1336 G C C A G A C T G T C G G C T G C C T T G A C T G C C C T C C C A C T C C T G A T G C T G A C C T T

hNTNRalpha.coding 1386 G G C C T T G T A G (SEQIDNO:2)
 rGDNFRalpha.coding 1356 G C T C A C C G C C C T T G C T G C C C T G T T A T C T G T A T C G T T G G C A G A A C G T C G T
 rNTNRalpha.coding 1386 G G C C T T G T A G (SEQIDNO:5)

rGDNFRalpha.coding 1406 A G (FROMSEQIDNO:20)

FIG. 3F

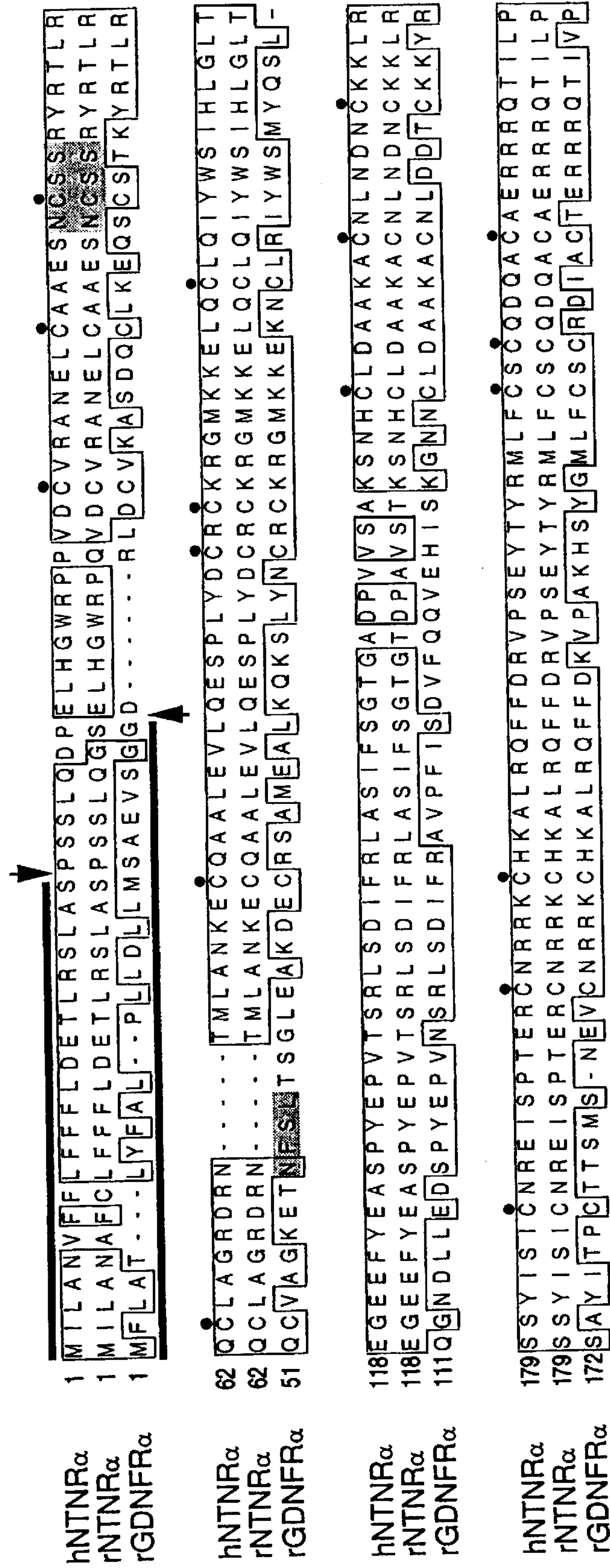


FIG. 4A

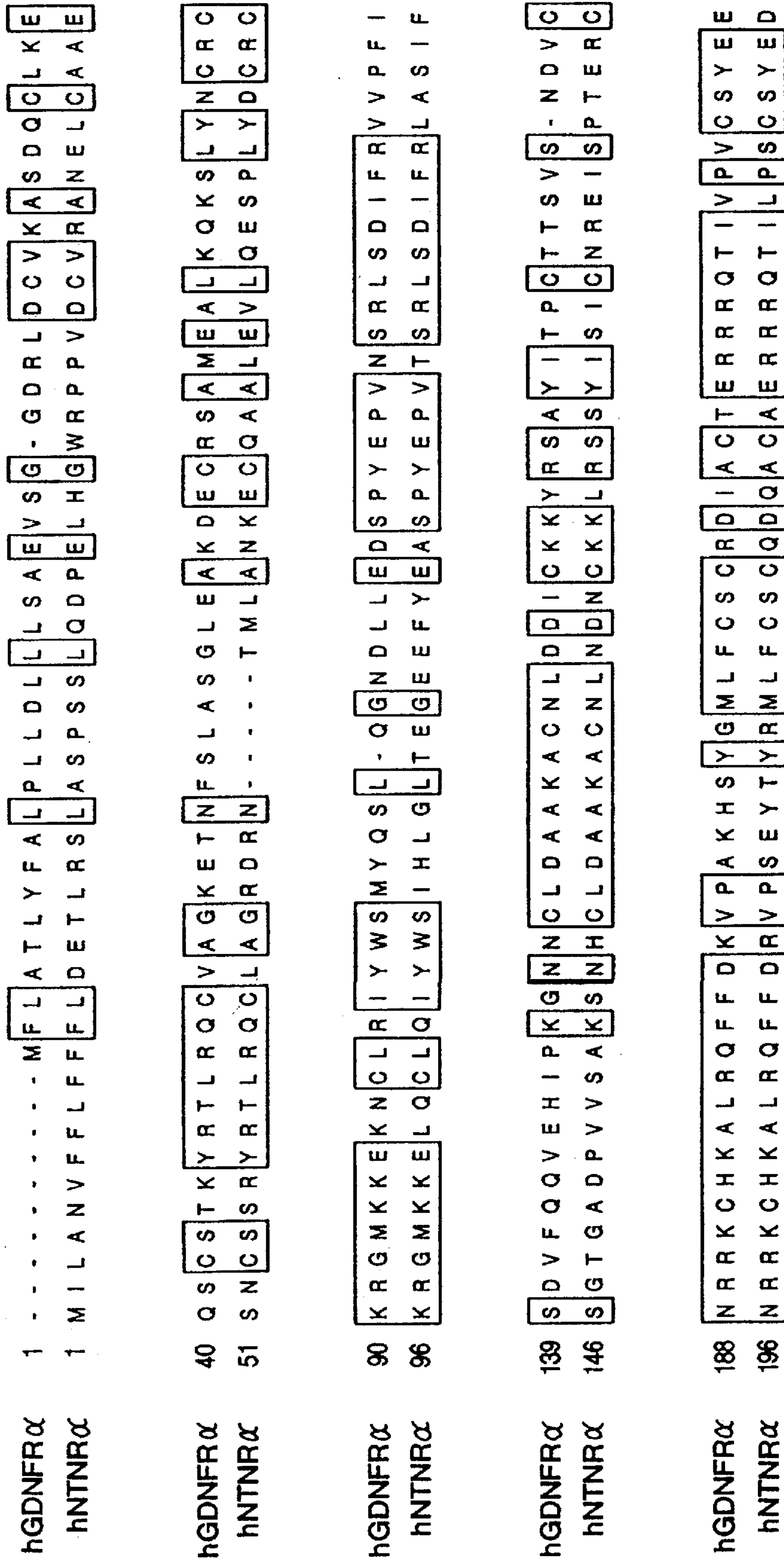


FIG. 5A

hGDNFR α 238 R E K P N C L S L Q D S C K T N Y I C R S R L A D F F T N C Q P E S R S V S C L K E N Y A D C L L
 hNTNR α 246 K E K P N C L D L R G V C R T D H L C R S R L A D F H A N C R A S Y Q T V T S C P A D N Y Q A C L G

hGDNFR α 288 A Y S G L I G T V M T P N Y I D S S - - S L S V A P W C D C S N S G N D L E E C L K F L N F F K D N
 hNTNR α 296 S Y A G M I G F D M T P N Y V D S S P T G I V S P W C S C R G S G N M E E E C E K F L R D F T E N

hGDNFR α 336 T C L K N A I Q A F G N G S D V T V W Q P A P P V Q T T A T T T T A L R V K N K P L G P A G S E N
 hNTNR α 346 P C L R N A I Q A F G N G T D V N V S P K G P S F Q A T Q A P R V E K T P S L P D D L S D S T S - -

hGDNFR α 386 E I P T H V L P P C A N L Q A Q K L K S N V S G N T H L C I S N G N Y E K E G L G A S S H I T T K S
 hNTNR α 394 - L G T S V I T T C T S V Q E Q G L K A N N S K E L S M C F T - - E L T T N I I P G S N K V I K P N

hGDNFR α 436 M A A P P S C G L S P L L V L V V T A L S T L L S L T E T S (SEQIDNO:22)
 hNTNR α 441 S G P S R A R P S A A L T V L S V L M L K L A L (SEQIDNO:3)

FIG. 5B

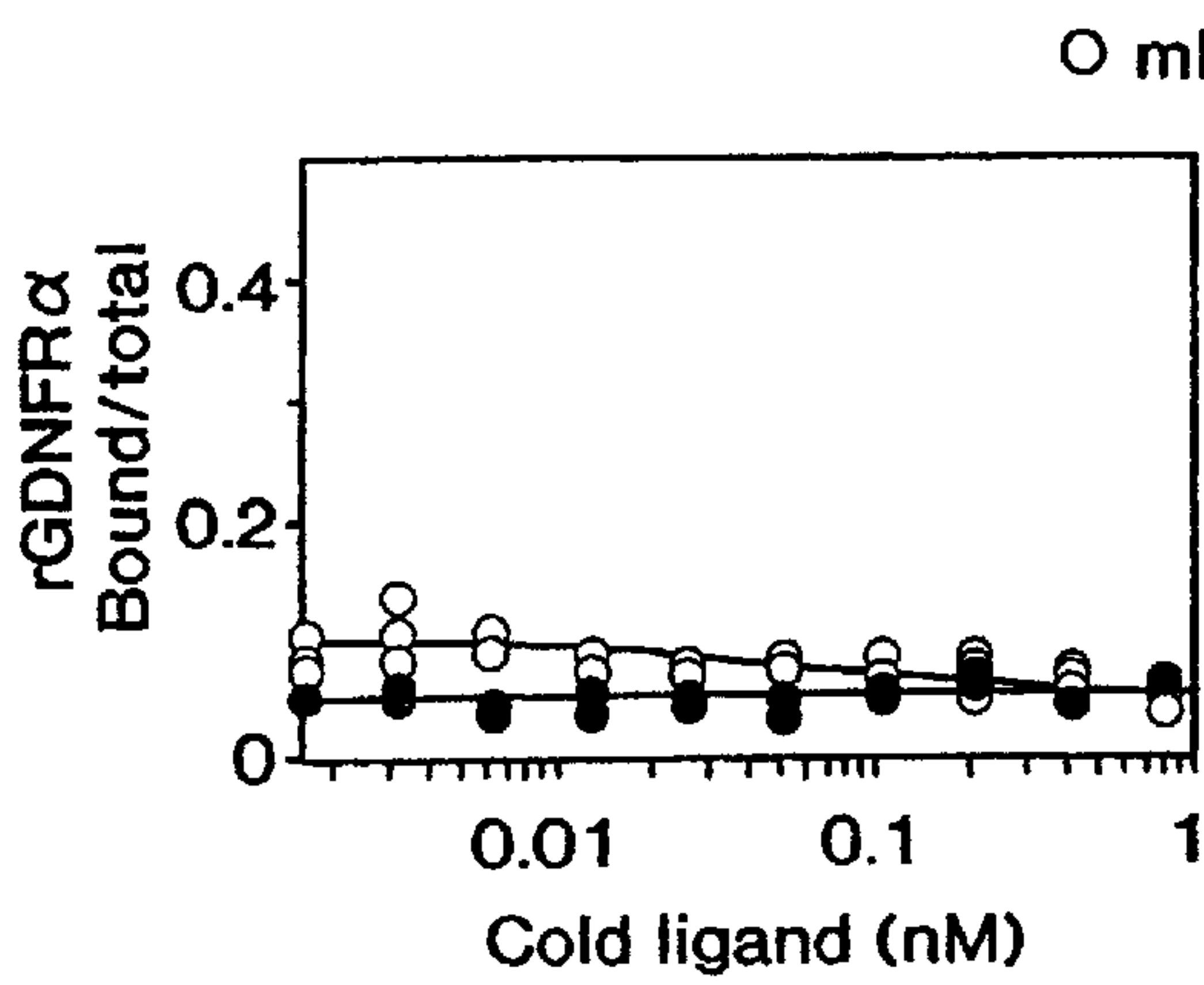


FIG. 6A

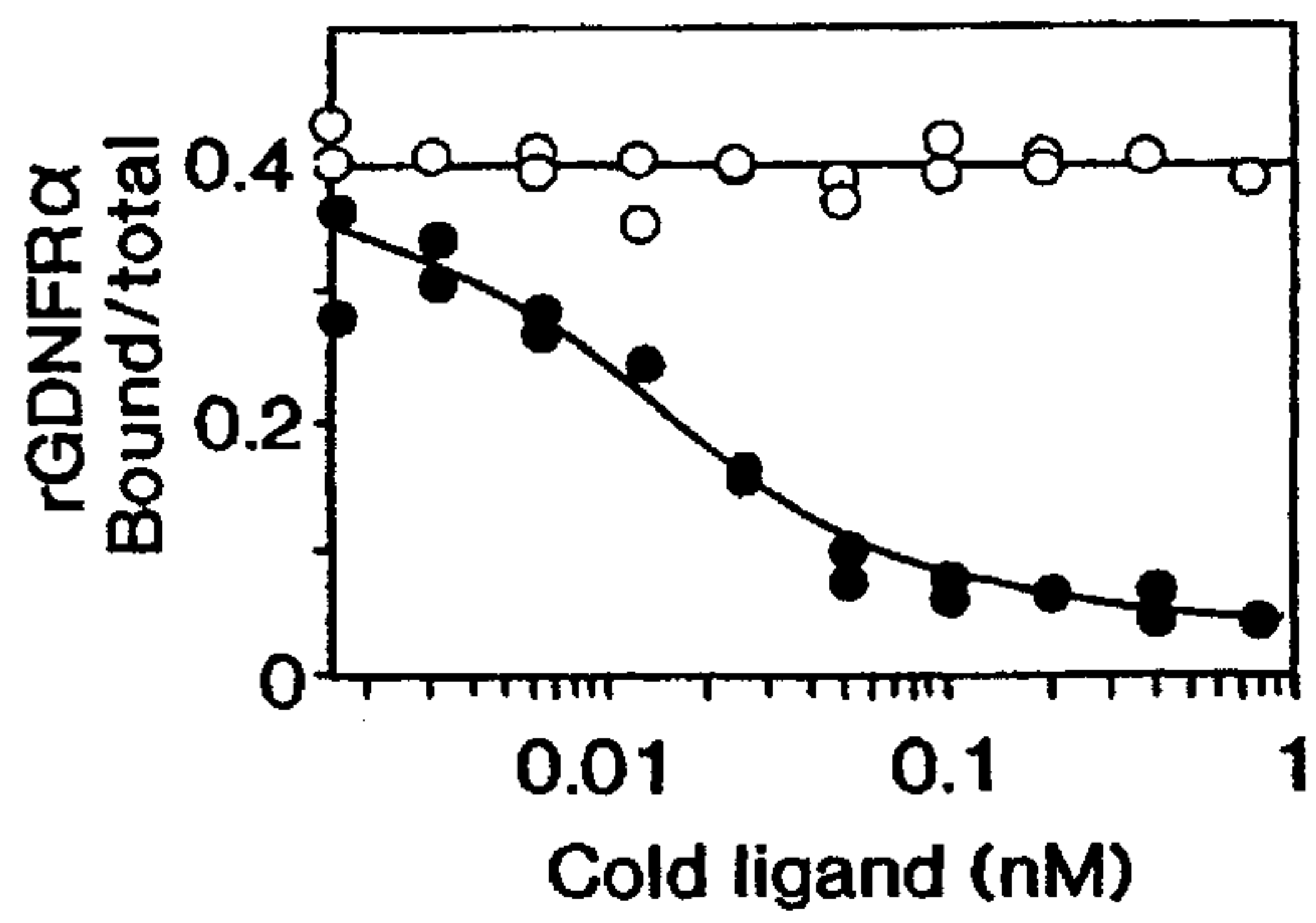


FIG. 6B

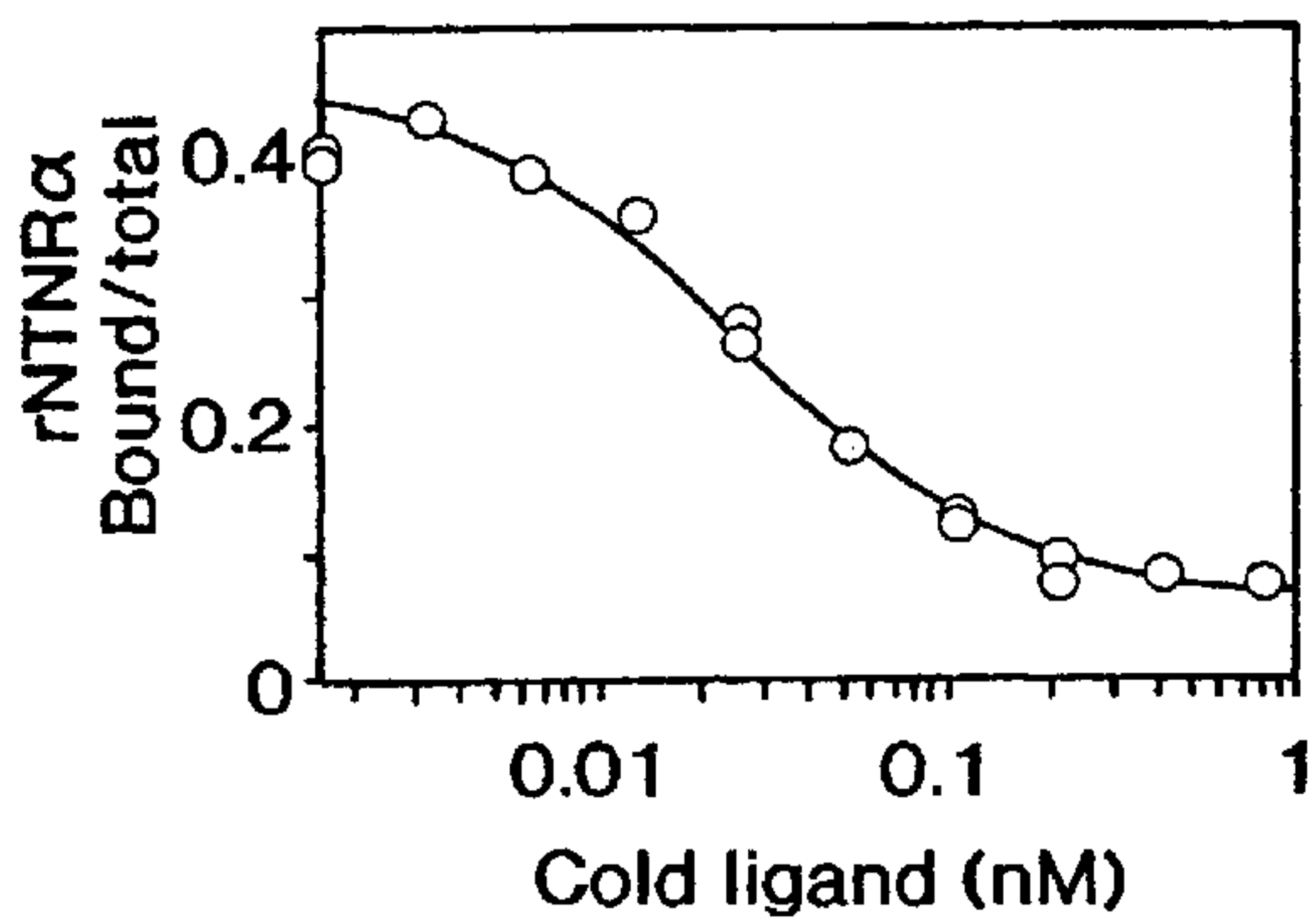


FIG. 6C

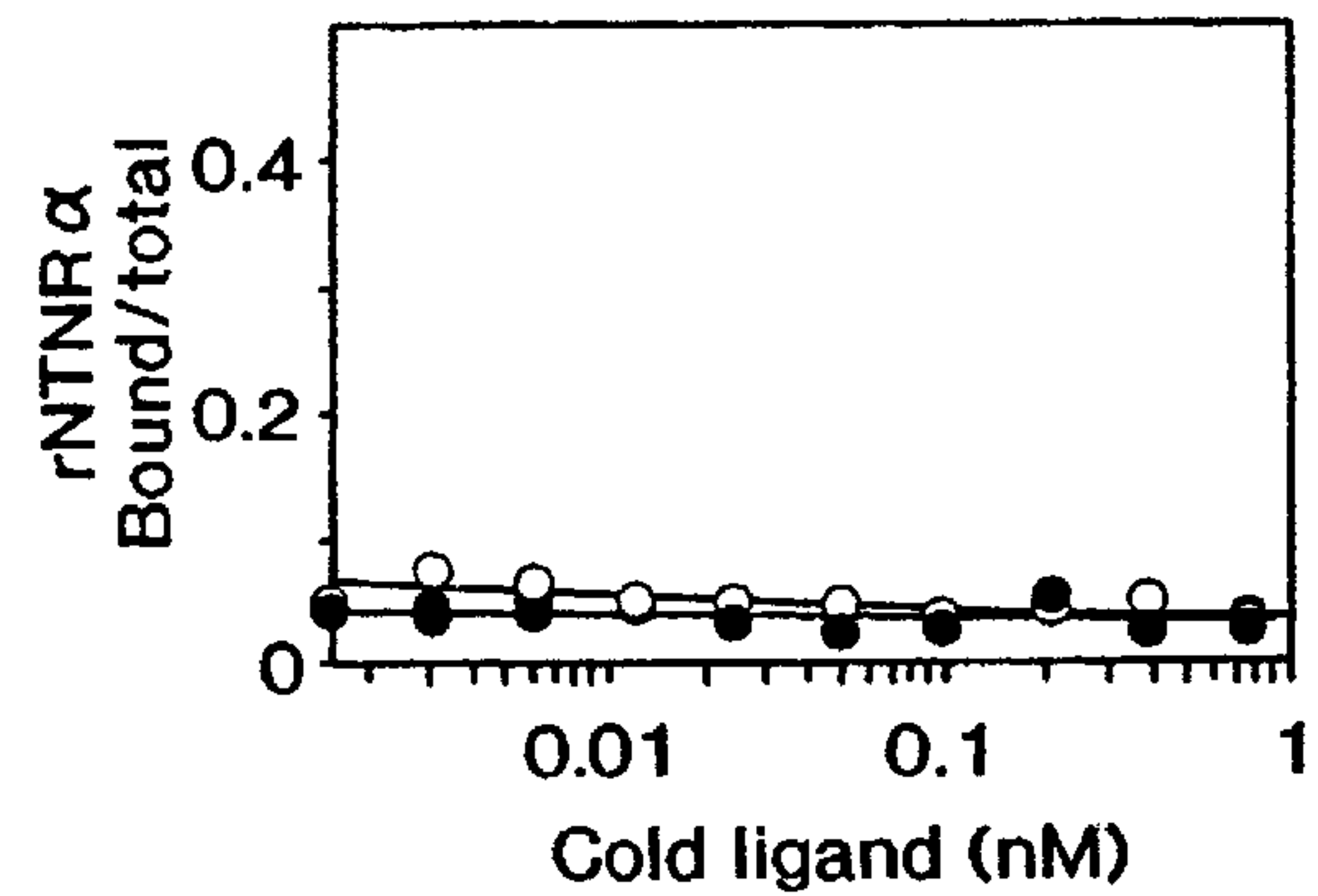


FIG. 6D

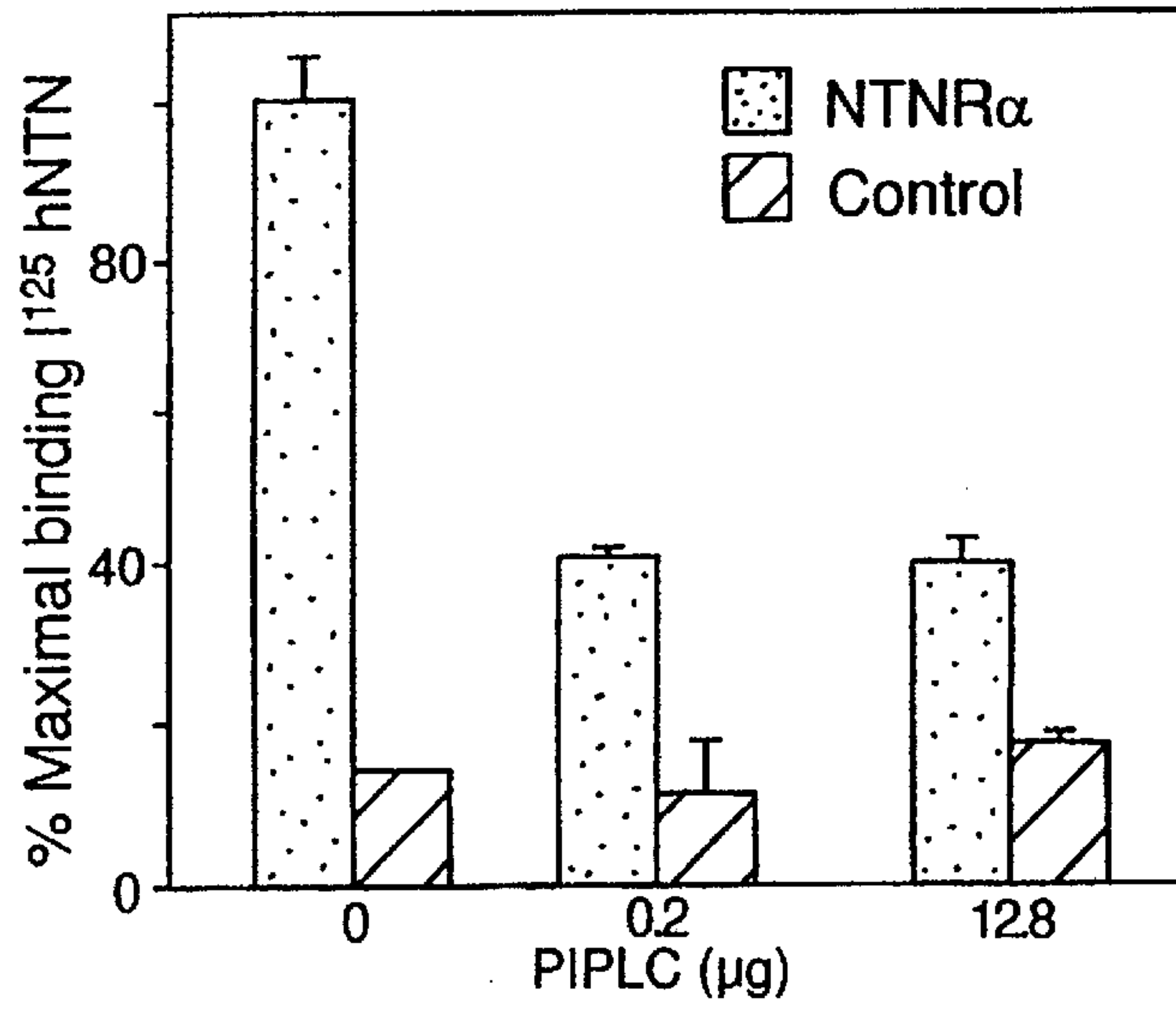


FIG. 7A

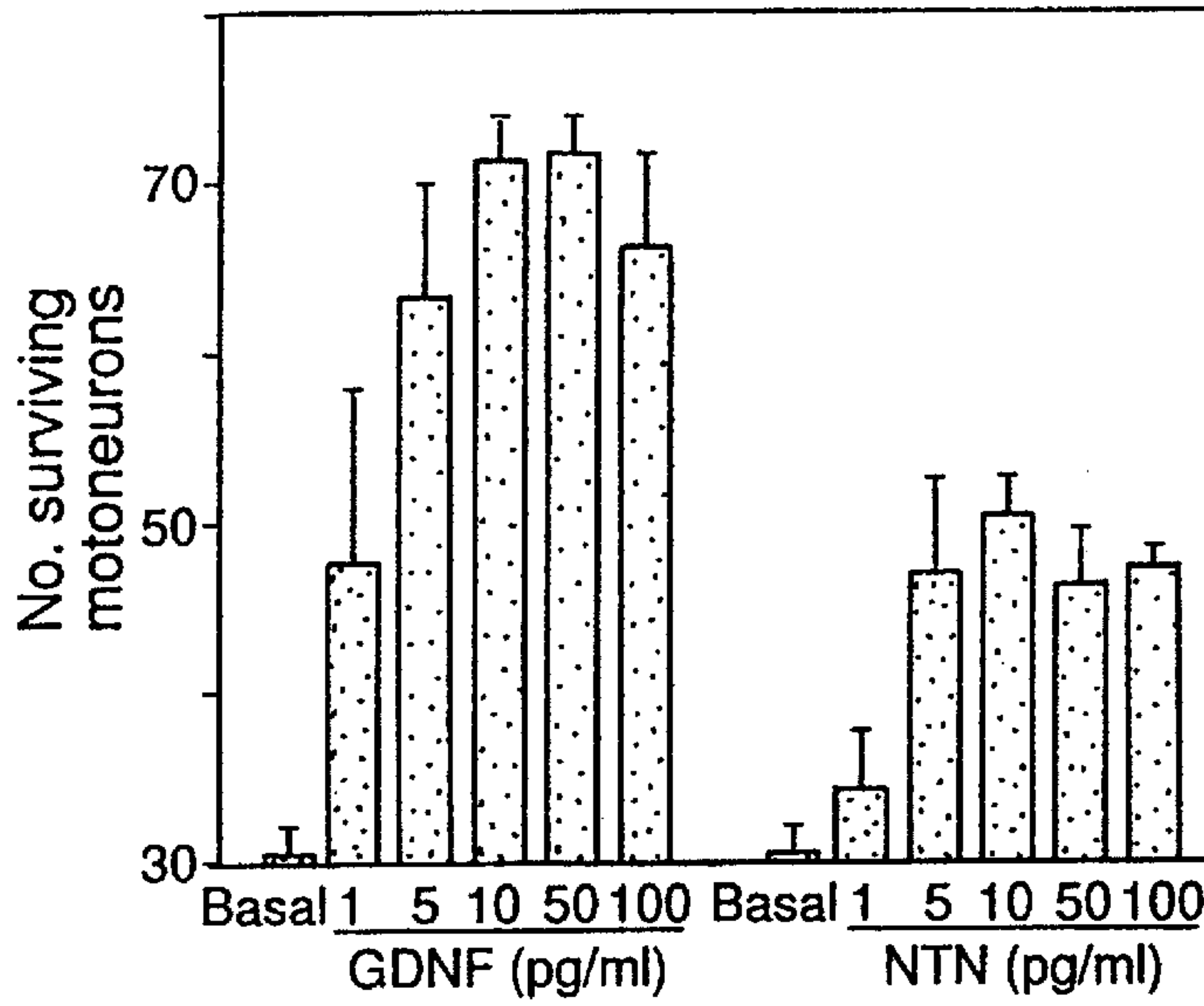


FIG. 7B

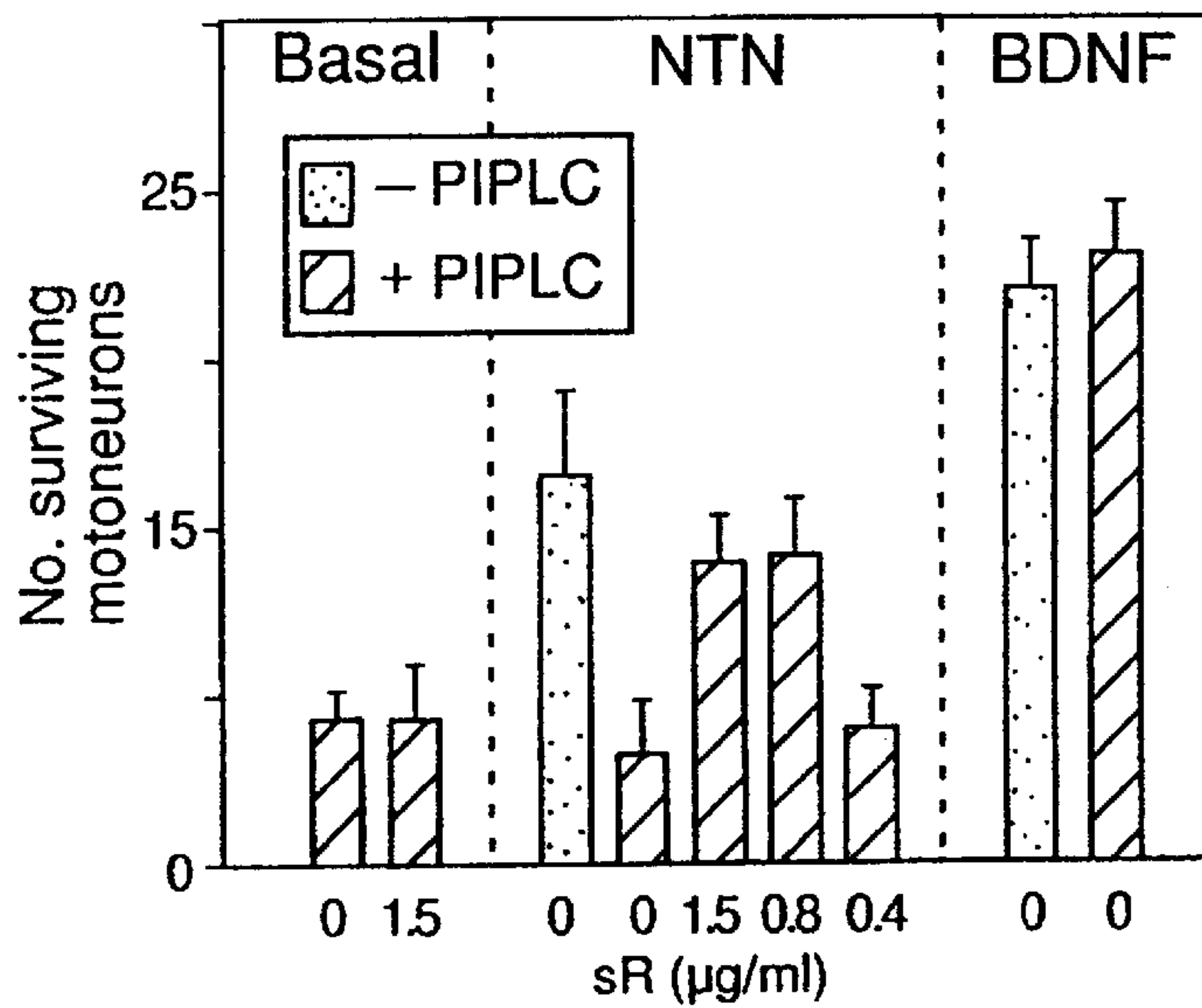


FIG. 7C

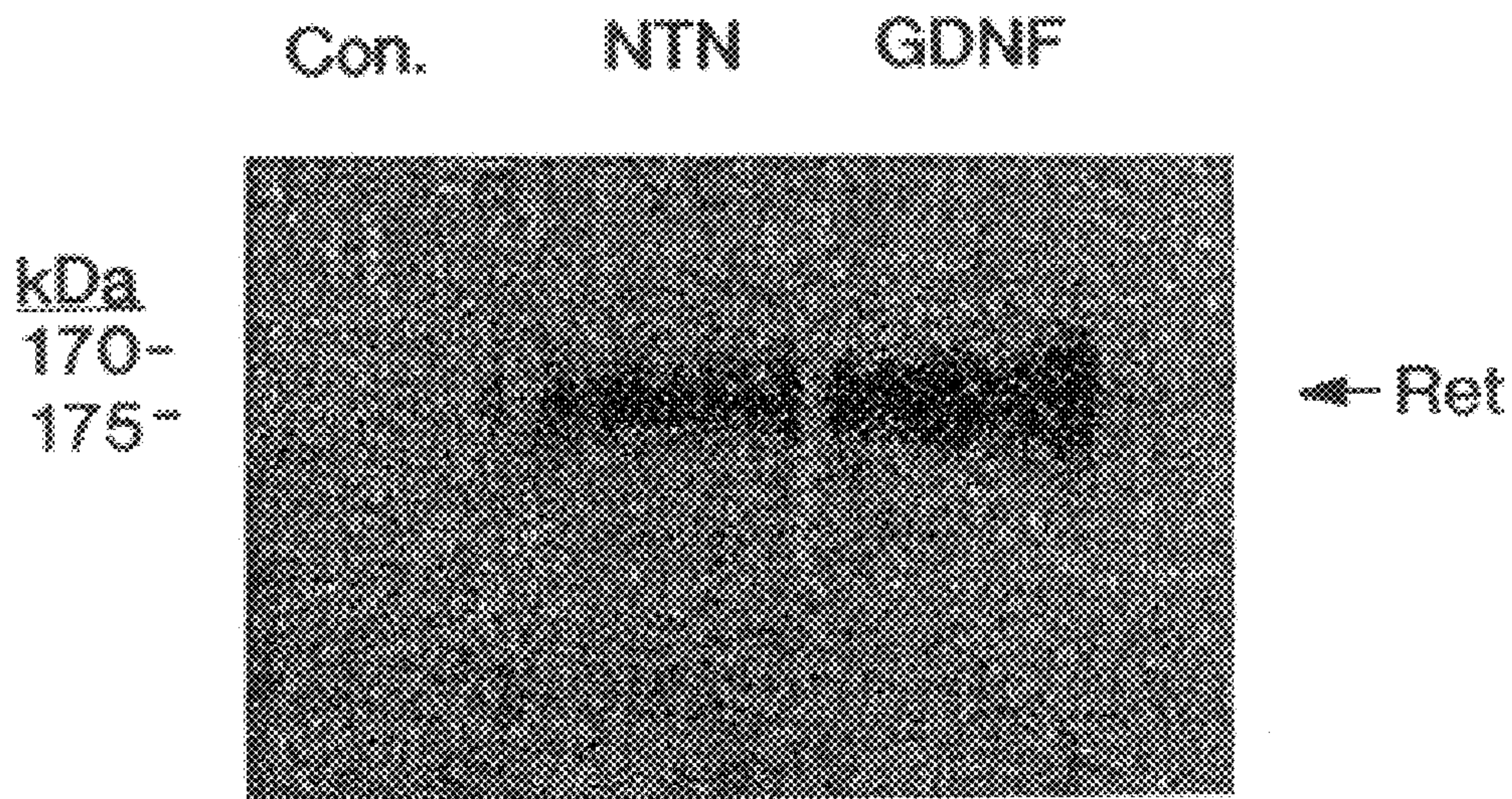


FIG. 7D

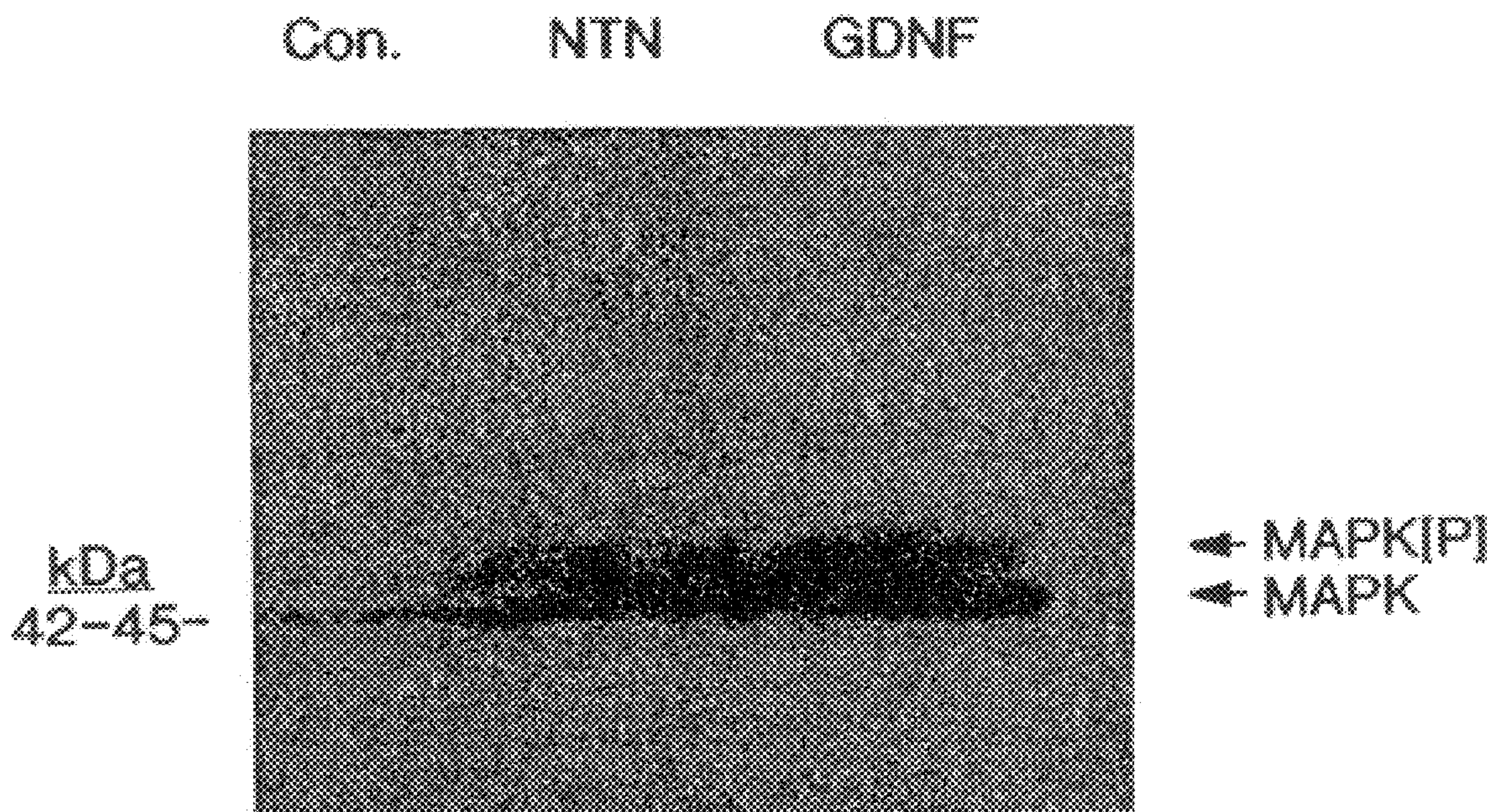


FIG. 7E

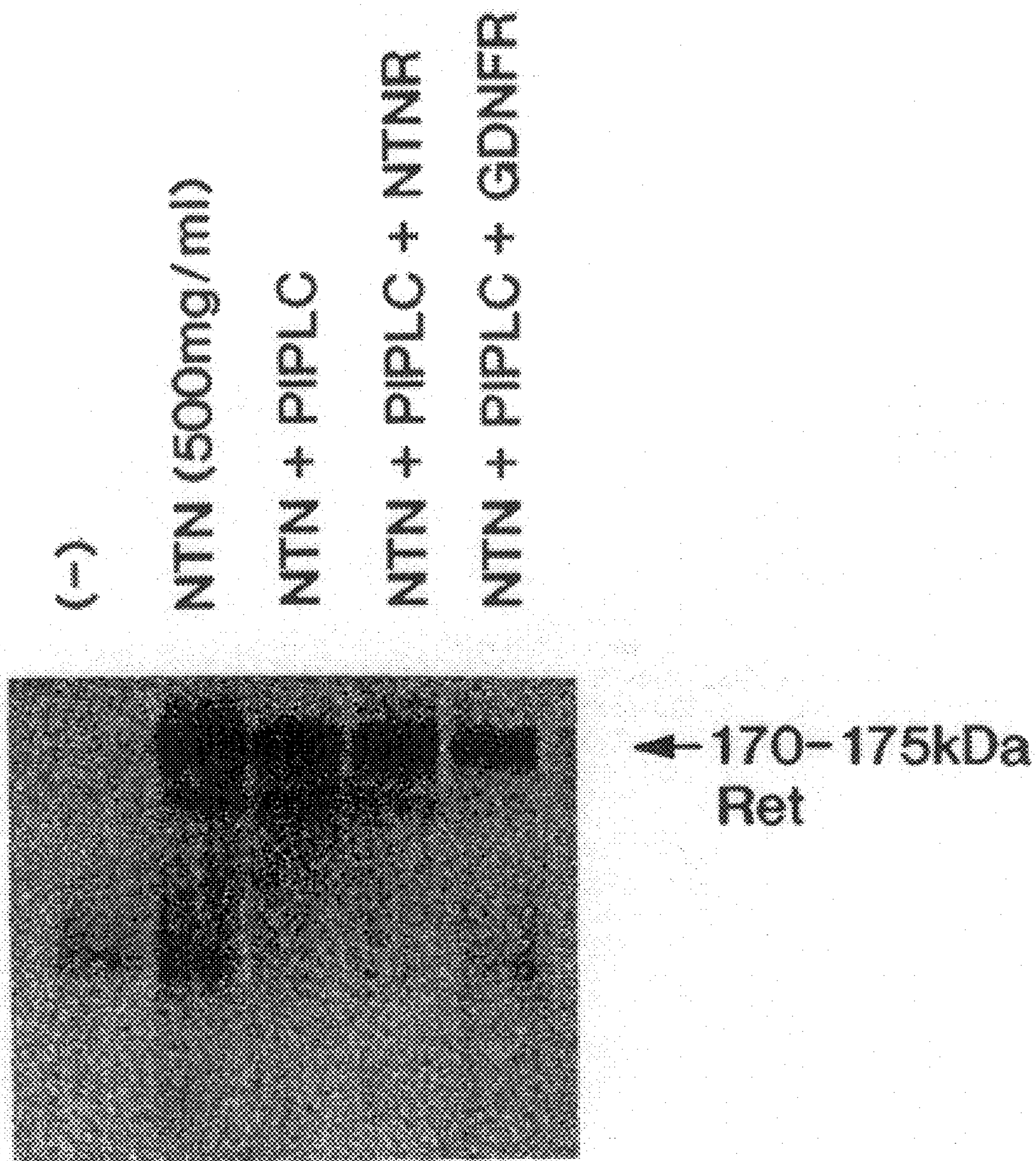


FIG. 7F

NEURTURIN RECEPTOR

TECHNICAL FIELD

The present invention relates to a Neurturin ("NTN") receptor designated NTNR α , and provides for NTNR α -encoding nucleic acid and amino acid sequences. In particular, the invention relates to native sequence NTNR α , NTNR α variants, soluble NTNR α variants including NTNR α extracellular domain, chimeric NTNR α , and antibodies which bind to the NTNR α (including agonist and neutralizing antibodies), as well as various uses for these molecules. It also relates to assay systems for detecting ligands to NTNR α , systems for studying the physiological role of NTN, diagnostic techniques for identifying NTN-related conditions, therapeutic techniques for the treatment of NTN-related and NTNR α -related conditions, and methods for identifying molecules homologous to NTNR α .

BACKGROUND

Neurotrophic factors such as insulin-like growth factors, nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, -4/5 and -6, ciliary neurotrophic factor, GDNF, and neurturin have been proposed as potential means for enhancing specific neuronal cell survival, for example, as a treatment for neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, stroke, epilepsy, Huntington's disease, Parkinson's disease, and peripheral neuropathy. It would be desirable to provide additional therapy for this purpose. Protein neurotrophic factors, or neurotrophins, which influence growth and development of the vertebrate nervous system, are believed to play an important role in promoting the differentiation, survival, and function of diverse groups of neurons in the brain and periphery. Neurotrophic factors are believed to have important signaling functions in neural tissues, based in part upon the precedent established with nerve growth factor (NGF). NGF supports the survival of sympathetic, sensory, and basal forebrain neurons both in vitro and in vivo. Administration of exogenous NGF rescues neurons from cell death during development. Conversely, removal or sequestration of endogenous NGF by administration of anti-NGF antibodies promotes such cell death (Heumann, *J. Exp. Biol.*, 132:133-150 (1987); Hefti, *J. Neurosci.*, 6:2155-2162 (1986); Thoenen et al., *Physiological Reviews* 60:1284-1335 (1980)).

Additional neurotrophic factors related to NGF have since been identified. These include brain-derived neurotrophic factor (BDNF) (Leibrock, et al., *Nature*. 341:149-152 (1989)), neurotrophin-3 (NT-3) (Kaisho, et al., *FEBS Lett.*, 266:187 (1990); Maisonpierre, et al., *Science*, 247:1446 (1990); Rosenthal, et al., *Neuron*, 4:767 (1990)), and neurotrophin 4/5 (NT-4/5) (Berkemeier, et al., *Neuron*, 7:857-866 (1991)).

Neurotrophins, similar to other polypeptide growth factors, affect their target cells through interactions with cell surface receptors. According to our current understanding, two kinds of transmembrane glycoproteins act as receptors for the known neurotrophins. Equilibrium binding studies have shown that neurotrophin-responsive neuronal cells possess a common low molecular weight (65,000-80,000 Daltons), a low affinity receptor typically referred to as p75^{LNGFR} or p75, and a high molecular weight (130,000-150,000 Dalton) receptor. The high affinity receptors are members of the trk family of receptor tyrosine kinases.

Receptor tyrosine kinases are known to serve as receptors for a variety of protein factors that promote cellular

proliferation, differentiation, and survival. In addition to the trk receptors, examples of other receptor tyrosine kinases include the receptors for epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF). Typically, these receptors span the cell membrane, with one portion of the receptor being intracellular and in contact with the cytoplasm, and another portion of the receptor being extracellular. Binding of a ligand to the extracellular portion of the receptor induces tyrosine kinase activity in the intracellular portion of the receptor, with ensuing phosphorylation of various intracellular proteins involved in cellular signaling pathways.

Glial cell line-derived neurotrophic factor ("GDNF") and Neurturin ("NTN") are two, recently identified, structurally related, potent survival factors for sympathetic sensory and central nervous system neurons (Lin et al. *Science* 260:1130-1132 (1993); Henderson et al. *Science* 266:1062-1064 (1994); Buj-Bello et al., *Neuron* 15:821-828 (1995); Kotzbauer et al. *Nature* 384:467-470 (1996)). Recently, GDNF was shown to mediate its actions through a multi-component receptor system composed of a ligand binding glycosyl-phosphatidyl inositol (GPI) linked protein (designated GDNFR α) and the transmembrane receptor tyrosine kinase Ret (Treanor et al. *Nature* 382:80-83 (1996); Jing et al. *Cell* 85:1113-1124 (1996); Trupp et al. *Nature* 381:785-789 (1996); Durbec et al. *Nature* 381:789-793 (1996)). The mechanism by which the NTN signal is transmitted has not been elucidated.

Aberrant expression of receptor tyrosine kinases ("RTK") correlates with transforming ability. For example, carcinomas of the liver, lung, breast and colon show elevated expression of Eph RTK. Unlike many other tyrosine kinases, this elevated expression can occur in the absence of gene amplification or rearrangement. Moreover, Hek, a human RTK, has been identified as a leukemia-specific marker present on the surface of a pre-B cell leukemia cell line. As with Eph, Hek also was overexpressed in the absence of gene amplification or rearrangements in, for example, hemopoietic tumors and lymphoid tumor cell lines. Overexpression of Myk-1 (a murine homolog of human Htk (Bennett et al., *J. Biol. Chem.*, 269(19):14211-8 (1994)) was found in the undifferentiated and invasive mammary tumors of transgenic mice expressing the Ha-ras oncogene. (Andres et al., *Oncogene*, 9(5):1461-7 (1994) and Andres et al., *Oncogene*, 9(8):2431 (1994)). Ret, the product of the c-ret proto-oncogene, is a member of the receptor tyrosine kinase superfamily.

In addition to their roles in carcinogenesis, a number of transmembrane tyrosine kinases have been reported to play key roles during development. Some receptor tyrosine kinases are developmentally regulated and predominantly expressed in embryonic tissues. Examples include Cek1, which belongs to the FGF subclass, and the Cek4 and Cek5 tyrosine kinases (Pasquale et al., *Proc. Natl. Acad. Sci., USA*, 86:5449-5453 (1989); Sajjadi et al., *New Biol.*, 3(8):769-78 (1991); and Pasquale, *Cell Regulation*, 2:523-534 (1991)). Eph family members are expressed in many different adult tissues, with several family members expressed in the nervous system or specifically in neurons (Maisonpierre et al., *Oncogene*, 8:3277-3288 (1993); Lai et al., *Neuron*, 6:691-704 (1991)).

The aberrant expression or uncontrolled regulation of any one of these receptor tyrosine kinases can result in different malignancies and pathological disorders. Therefore, there exists a need to identify means to regulate, control and manipulate receptor tyrosine kinases ("RTK") and their associated ligands or GPI-linked receptors, in order to

provide new and additional means for the diagnosis and therapy of receptor tyrosine kinase pathway-related disorders and cellular processes. The present application provides the clinician and researcher with such means by providing new molecules that are specific for interacting with certain RTK receptors. These compounds and their methods of use, as provided herein, allow exquisite therapeutic control and specificity. Accordingly, it is an object of the present invention to provide an improved therapy for the prevention and/or treatment of neurological conditions and other conditions in which certain neurotrophic signaling pathways play a role.

These and other objects of the invention will be apparent to the ordinarily skilled artisan upon consideration of the specification as a whole.

SUMMARY

A NTN receptor termed NTNR α , a soluble form of the receptor, and a NTNR α extracellular domain ("ECD") are disclosed herein. Also disclosed are NTNR α polypeptides, optionally conjugated with or fused to molecules which increase the serum half-lives thereof, and optionally formulated as pharmaceutical compositions with a physiologically acceptable carrier.

Soluble NTNR α that retains both ligand binding, preferably NTN binding, and receptor signaling function (via Ret receptor tyrosine kinase) can be used to impart, restore, or enhance NTNR α -ligand (preferably NTN) responsiveness to cells. This responsiveness includes ligand-binding, Ret tyrosine phosphorylation and Ret-mediated downstream activity, which can result in modulation of cell activity such as survival or growth. The embodiments find use in vivo, in vitro or ex vivo. NTNR α ECD that binds NTN can be used as an antagonist to NTN ligand to reduce activation of endogenous NTNR α . This is useful in conditions characterized by excess levels of NTN ligand and/or excess NTNR α activation in a mammal.

Pharmaceutical compositions of soluble NTNR α , preferably ECD, further include an NTNR α ligand, preferably NTN. Such compositions are useful where it is desirable to prolong the half-life of the ligand, provide slow, sustained release of ligand, impart NTNR α -ligand responsiveness to a target cell, and/or activate or enhance endogenous cellular NTNR α or Ret activity directly. Optionally, the composition further contains one or more cytokines, neurotrophic factors, or their agonist antibodies.

Chimeric NTNR α molecules such as NTNR α immunoadhesin (having long serum half-lives) and epitope-tagged NTNR α are disclosed. These find particular use as soluble forms of NTNR α . Immunoadhesins can also be employed as NTNR α antagonists in conditions or disorders in which neutralization of NTNR α biological activity is beneficial. Bispecific immunoadhesins (for example, combining a NTNR α -ligand binding activity with a ligand-binding domain of another cytokine or neurotrophic factor receptor) can form high affinity binding complexes for NTNR α -ligands in combination with other factors or for targeted delivery.

Also provided are methods for identifying a molecule which binds to and/or activates NTNR α . Thus assays are provided to screen for or identify NTNR α -ligand molecules (such as peptides, antibodies, and small molecules) that are agonists or antagonists of NTNR α . Such methods generally involve exposing an immobilized NTNR α to a molecule suspected of binding thereto and determining binding of the molecule to the immobilized NTNR α and/or evaluating

whether or not the molecule activates (or blocks activation of) the NTNR α . In order to identify such NTN ligands, the NTNR α can be expressed on the surface of a cell and used to screen libraries of synthetic candidate compounds or naturally-occurring compounds (e.g., from endogenous sources such as serum or cells). NTNR α can also be used as a diagnostic tool for measuring serum levels of endogenous or exogenous NTNR α -ligand.

In a further embodiment, a method for purifying an NTNR α -ligand is provided. This finds use in commercial production and purification of therapeutically active molecules that bind to this receptor. In one embodiment the molecule of interest (generally in a composition comprising one or more contaminants) is adsorbed to immobilized NTNR α (e.g., NTNR α immunoadhesin immobilized on a protein A resin). The contaminants, by virtue of their inability to bind to the NTNR α , will generally not bind the resin. Accordingly, it is then possible to recover the molecule of interest from the resin by changing the elution conditions, such that the ligand molecule is released from the immobilized receptor.

Antibodies are provided that specifically bind to NTNR α . Preferred antibodies are monoclonal antibodies that are non-immunogenic in a human and bind to an epitope in the extracellular domain of the receptor. Preferred antibodies bind the NTNR α with an affinity of at least about 10^6 L/mole, more preferably 10^7 L/mole. Preferred antibodies are agonist antibodies.

Antibodies, which bind to NTNR α , can be optionally fused to a heterologous polypeptide. The antibody or fusion finds particular use to isolate and purify NTNR α from a source of the receptor.

In a further aspect is provided a method for detecting NTNR α in vitro or in vivo which includes the steps of contacting an NTNR α antibody with a sample suspected of containing the receptor, and detecting if binding has occurred.

For certain applications it is desirable to have an agonist antibody. Such agonist antibodies are useful for activating NTNR α as described for NTNR α -ligands such as NTN. Furthermore, these antibodies are useful to treat conditions in which an effective amount of NTNR α activation leads to a therapeutic benefit in the mammal. For example, the agonist antibody can be used to elicit an NTN response in a cell comprising NTNR α and, preferably, Ret. For therapeutic applications it is desirable to prepare a composition having the agonist antibody and a physiologically acceptable carrier. Optionally, the composition further contains one or more cytokines, neurotrophic factors, or their agonist antibodies.

In other embodiments, the antibody is a neutralizing antibody. Such molecules can be used to treat conditions characterized by unwanted or excessive activation of NTNR α .

In addition to the above, the invention provides isolated nucleic acid molecules, expression vectors and host cells encoding NTNR α which can be used in the recombinant production of NTNR α as described herein. The isolated nucleic acid molecules and vectors are also useful to prepare transgenic animals, for gene therapy applications to treat patients with NTNR α defects or increase responsiveness of cells to NTNR α ligands, or alternatively to decrease NTNR α activity (as by use of antisense nucleic acid).

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1C depict the nucleic acid sequence (SEQ ID NO.: 1) of the sense strand of the cDNA encoding full length

human NTN α , the hNTN α -encoding sequence (SEQ ID NO.: 2), and the deduced amino acid sequence of full length hNTN α (SEQ ID NO.: 3). Nucleotides are numbered at the beginning of the sense strand. Amino acid residues are numbered at the beginning of the amino acid sequence.

FIGS. 2A–2D depict the nucleic acid sequence (SEQ ID NO.: 4) of the sense strand of the cDNA encoding full length rat NTN α , the rNTN α -encoding sequence (SEQ ID NO.: 5), and the deduced amino acid sequence of full length rNTN α (SEQ ID NO.: 6). Nucleotides are numbered at the beginning of the sense strand. Amino acid residues are numbered at the beginning of the amino acid sequence.

FIGS. 3A–3F compare hNTN α -, rNTN α -, and rGDNFR α -encoding (from SEQ ID NO: 20) nucleic acids.

FIGS. 4A–4B are a comparison of the hNTN α , rNTN α , and rGDNFR α proteins, with features indicated. Signal peptides are indicated by a solid line. Signal cleavage sites are marked with arrows. Potential glycosylation sites are shaded. The hydrophobic domain of the GPI attachment site is doubly underlined. The small amino acid residues that constitute a cleavage/attachment site for GPI-linked proteins are marked with asterisks. Consensus cysteine residues are indicated by a solid circle. The extracellular domain (“ECD”) is flanked by the signal peptide and the GPI-attachment site.

FIGS. 5A–5B are a comparison of the amino acid sequences of hNFNR α and hGDNFR α .

FIGS. 6A–6D depict binding of I¹²⁵ NTN and GDNF to NTN α - or GDNFR α -expressing cells and displacement by unlabeled NTN. FIGS. 6A and 6C show the binding of ¹²⁵I mouse NTN (¹²⁵I-mNTN) to rat GDNFR α (“rGDNFR α ”) or rat NTN α (“rNTN α ”), respectively. FIGS. 6B and 6D show the binding of ¹²⁵IrGDNF (¹²⁵I-rGDNF) to rat GDNFR α (“rGDNFR α ”) or rat NTN α (“rNTN α ”), respectively. As depicted by the Scatchard analysis, displayed in the inset of FIG. 6B, GDNF binds GDNFR α with a K_d value of 3 pM. A similar K_d was reported in a cell based assay (Jing et al. *Cell* 85:1 113–1124 (1996)). Mouse NTN binds rNTN α with a K_d value of 10 pM (see inset to FIG. 6C). Human NTN α displayed a similar binding specificity as rat NTN α (data not shown). Although in these experiments no binding of ¹²⁵I NTN to GDNFR α (FIG. 6A) and of ¹²⁵IrGDNF to NTN α (FIG. 6D) were detected, experiments performed with biotinylated NTN and GDNF revealed low affinity binding (K_d above 1 mM) of NTN to GDNFR α , and vice versa.

FIGS. 7A–7F depict interaction between NTN, NTN α and Ret. FIG. 7A depicts binding of ¹²⁵I NTN to cells expressing NTN α . Consistent with the prediction that NTN α is a GPI-linked protein, binding of ¹²⁵I NTN to NTN α expressing cells was reduced by 50–70% following treatment with PIPLC. FIG. 7B depicts survival response of embryonic, rat spinal motoneurons to GDNF or NTN. In agreement with its receptor distribution, NTN is a potent survival factor for spinal motoneurons. FIG. 7C depicts survival response of embryonic, rat spinal motoneurons to NTN or BDNF in the presence of PIPLC and a soluble NTN α . PIPLC treatment reduced the survival response to NTN by 50–90% without changing the response to BDNF. Soluble NTN α (s α) restores the response of PIPLC-treated motoneurons to NTN. FIG. 7D depicts NTN induction of tyrosine phosphorylation of Ret in neuroblastoma TGW-1 cells. FIG. 7E depicts NTN induction of phosphorylation of ERK in TGW-1. FIG. 7F depict the NTN-responsiveness (e.g., Ret phosphorylation) imparted by an NTN-soluble NTN α complex to Ret-expressing cells.

Legends: (Con)=untransfected cells. (Ret)=cells transfected with Ret alone. (R α +Ret)=cells transfected with Ret and NTN α . In all cases, cells were exposed to NTN (100 ng/ml) and then processed for immunoprecipitation with NTN antisera.

DETAILED DESCRIPTION

In describing the present invention, the following terms will be employed and are intended to be defined as indicated below.

The terms “NTN α ” or “NTN α polypeptide” when used herein encompass native sequence NTN α ; NTN α variants; NTN α extracellular domain; and chimeric NTN α . (each of which is defined herein). Optionally, the NTN α is not associated with native glycosylation. “Native glycosylation” refers to the carbohydrate moieties which are covalently attached to NTN α when it is produced in the mammalian cell from which it is derived in nature. Accordingly, human NTN α produced in a non-human cell is an example of a NTN α which may “not be associated with native glycosylation.” Sometimes, the NTN α is unglycosylated (e.g., as a result of being produced recombinantly in a prokaryote).

A “native sequence NTN” comprises a polypeptide having the same amino acid sequence as a NTN α derived from nature. Thus, a native sequence NTN α can have the amino acid sequence of naturally occurring rat NTN α , murine NTN α , human NTN α , or NTN α from any other mammalian species. Such native sequence NTN α polypeptides can be isolated from nature or can be produced by recombinant or synthetic means. The term “native sequence NTN” specifically encompasses naturally-occurring truncated forms of the NTN α , naturally-occurring variant forms (e.g., alternatively spliced forms), and naturally-occurring allelic variants of the NTN α . The preferred native sequence NTN α is a mature native sequence NTN α . NTN α sequence for human and rat are shown in FIGS. 1A–1C and 2A–2D. Preferred molecules are those comprising a nucleic acid molecule that is capable of hybridizing under moderate, and more preferably under stringent hybridization conditions, with the DNA sequence encoding the human NTN receptor shown in FIGS. 1A–1C. In one embodiment the NTN nucleic acid hybridizes at 42° C. in 20% formamide with the DNA sequence encoding the NTN receptor shown in FIGS. 1A–1C. In another embodiment a NTN nucleic acid molecule is capable of hybridizing at 42° C. in 20% formamide with a DNA sequence of at least 10 contiguous bases, and preferably at least 20 contiguous bases, more preferably with at least 45 bases, and even more preferably with at least 60 bases encoding a portion of the complete NTN receptor shown in FIGS. 1A–1C or 2A–2D. Preferred sequences do not hybridize GDNFR α sequences under similar conditions.

The “NTN α extracellular domain” (ECD) is a form of the NTN α which is essentially free of the transmembrane and cytoplasmic domains of NTN α , i.e., has less than 1% of such domains, preferably 0.5 to 0% of such domains, and more preferably 0.1 to 0% of such domains. Ordinarily, the NTN α ECD will have an amino acid sequence having at least about 60% amino acid sequence identity with the amino acid sequence of the ECD of an NTN α , for example as indicated in FIGS. 1A–1C or 2A–2D for NTN α or the corresponding sequences provided herein, e.g. mouse sequences, preferably at least about 65%, more preferably at least about 75%, even more preferably at least about 80%, even more preferably at least about 90%, with increasing

preference of 95%, to at least 99% amino acid sequence identity, and finally to 100% identity, and thus includes NTN α variants as defined below. Preferred sequences will be at least 16 amino acids long, preferably at least 20 amino acids long, and even more preferably at least 40 amino acids long.

“NTN α variant” means a biologically active NTN α as defined below having less than 100% sequence identity (but at least 60% identity) with a NTN α , for example, having the deduced amino acid sequence shown in FIGS. 1A–1C or 2A–2D for NTN α or with the sequences provided herein. Such NTN α variants include NTN α polypeptides wherein one or more amino acid residues are added at the N- or C-terminus of, or within, a NTN α sequence; from about one to thirty amino acid residues are deleted, and optionally substituted by one or more amino acid residues; and derivatives of the above polypeptides, wherein an amino acid residue has been covalently modified so that the resulting product has a non-naturally occurring amino acid. Ordinarily, a biologically active NTN α variant will have an amino acid sequence having about 60% amino acid sequence identity with the amino acid sequence of a naturally-occurring NTN α (e.g., as shown in FIGS. 1A–1C or 2A–2D or the corresponding sequences provided herein), preferably at least about 65%, more preferably at least about 75%, even more preferably at least about 80%, even more preferably at least about 90%, with increasing preference of 95%, to at least 99% amino acid sequence identity, and finally to 100% identity.

A “chimeric NTN α ” is a polypeptide comprising full-length NTN α or one or more domains thereof (e.g., the extracellular domain) fused or bonded to heterologous polypeptide. The chimeric NTN α will generally share at least one biological property in common with NTN α . Examples of chimeric NTN α include immunoadhesins and epitope-tagged NTN α .

The term “immunoadhesin” is used interchangeably with the expression “NTN α -immunoglobulin chimera” and refers to a chimeric molecule that combines a portion of the NTN α (generally the extracellular domain thereof) with an immunoglobulin sequence. The immunoglobulin sequence preferably, but not necessarily, is an immunoglobulin constant domain. The immunoglobulin moiety in the chimeras of the present invention may be obtained from IgG1, IgG2, IgG3 or IgG4 subtypes, IgA, IgE, IgD or IgM, but preferably IgG1 or IgG3.

The term “epitope-tagged” when used herein refers to a chimeric polypeptide comprising NTN α fused to a “tag polypeptide”. The tag polypeptide has enough residues to provide an epitope against which an antibody thereagainst can be made, yet is short enough such that it does not interfere with biological activity of the NTN α . The tag polypeptide preferably also is fairly unique so that the antibody thereagainst does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8–50 amino acid residues (preferably between about 9–30 residues). Preferred are poly-histidine sequences, which bind nickle, allowing isolation of the tagged protein by Ni-NTA chromatography as described (Lindsay et al. *Neuron* 17:571–574 (1996)), for example.

“Isolated NTN α ” means NTN α that has been purified from a NTN α source or has been prepared by recombinant or synthetic methods and is sufficiently free of other peptides or proteins (1) to obtain at least 15 and preferably 20 amino acid residues of the N-terminal or of an internal amino acid

sequence by using a spinning cup sequenator or the best commercially available amino acid sequenator marketed or as modified by published methods as of the filing date of this application, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Homogeneity here means less than about 5% contamination with other source proteins.

“Essentially pure” protein means a composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight. “Essentially homogeneous” protein means a composition comprising at least about 99% by weight of protein, based on total weight of the composition.

“Biological property” when used in conjunction with either “NTN α ” or “isolated NTN α ” means having an effector or antigenic function or activity that is directly or indirectly caused or performed by native sequence NTN α (whether in its native or denatured conformation). Effector functions include ligand binding, and enhancement of survival, differentiation and/or proliferation of cells (especially proliferation of cells). However, effector functions do not include possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against native sequence NTN α .

An “antigenic function” means possession of an epitope or antigenic site that is capable of cross-reacting with antibodies raised against native sequence NTN α . The principal antigenic function of a NTN α polypeptide is that it binds with an affinity of at least about 10^6 L/mole to an antibody raised against native sequence NTN α . Ordinarily, the polypeptide binds with an affinity of at least about 10^7 L/mole. The antibodies used to define “antigenic function” are rabbit polyclonal antibodies raised by formulating the NTN α in Freund’s complete adjuvant, subcutaneously injecting the formulation, and boosting the immune response by intraperitoneal injection of the formulation until the titer of the anti-NTN α antibody plateaus.

“Biologically active” when used in conjunction with either “NTN α ” or “isolated NTN α ” means a NTN α polypeptide that exhibits or shares an effector function of native sequence NTN α and that may (but need not), in addition, possess an antigenic function. A principal effector function of the NTN α is its ability to bind NTN. Another principal effector function of NTN α is activating Ret tyrosine kinase (resulting in Ret autophosphorylation) to activate downstream pathways mediated by Ret signaling function.

“Antigenically active” NTN α is defined as a polypeptide that possesses an antigenic function of NTN α and that may (but need not) in addition possess an effector function.

“Percent amino acid sequence identity” with respect to the NTN α sequence is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the residues in the NTN α sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. None of N-terminal, C-terminal, or internal extensions, deletions, or insertions into the candidate NTN α sequence shall be construed as affecting sequence identity or homology.

“NTN ligand” is a molecule which binds to and preferably activates native sequence NTN α . The ability of a molecule to bind to NTN α can be determined, for example, by the ability of the putative ligand to bind to NTN α immunoadhesin coated on an assay plate, for example. Specificity of

binding can be determined by comparing binding to GDNFR α . Competitive binding of NTN to NTN α is a preferred property of the ligand. The thymidine incorporation assay provides another means for screening for ligands which activate NTN α function.

A "thymidine incorporation assay" can be used to screen for molecules which activate the NTN α . In order to perform this assay, IL-3 dependent Baf3 cells (Palacios et al., *Cell*, 41:727-734 (1985)) are stably transfected with full length native sequence NTN α as described herein and Ret. The NTN α /Ret/Baf3 cells so generated are starved of IL-3 for 24 hours in a humidified incubator at 37° C. in 5% CO₂ and air. Following IL-3 starvation, the cells are plated out in 96 well culture dishes with, or without, a test sample containing a potential agonist (such test samples are optionally diluted) and cultured for 24 hours in a cell culture incubator. 20 μ l of serum free RPMI media containing 1 μ Ci of ³H thymidine is added to each well for the last 6-8 hours. The cells are then harvested in 96 well filter plates and washed with water. The filters are then counted using a Packard Top Count Microplate Scintillation Counter, for example. Agonists are expected to induce a statistically significant increase (to a P value of 0.05) in ³H uptake, relative to control. Preferred agonists leads to an increase in ³H uptake which is at least two fold of that of the control. Other assays are described herein.

An "isolated" NTN α nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the NTN α nucleic acid. An isolated NTN α nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated NTN α nucleic acid molecules therefore are distinguished from the NTN α nucleic acid molecule as it exists in natural cells. However, an isolated NTN α nucleic acid molecule includes NTN α nucleic acid molecules contained in cells that ordinarily express NTN α where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, a ribosome binding site, and possibly, other as yet poorly understood sequences. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants"

and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies, antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature*, 256: 495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567 (Cabilly et al.)). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson et al., 624-628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581-597 (1991), for example.

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (Cabilly et al., supra; Morrison et al., *Proc. Natl. Acad. Sci. USA*, 81:6851-6855(1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may

comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature*, 321:522-525 (1986); Reichmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

“Non-immunogenic in a human” means that upon contacting the polypeptide of interest in a physiologically acceptable carrier and in a therapeutically effective amount with the appropriate tissue of a human, no state of sensitivity or resistance to the polypeptide of interest is demonstrable upon the second administration of the polypeptide of interest after an appropriate latent period (e.g., 8 to 14 days).

By “agonist antibody” is meant an antibody which is a NTNR α ligand, able to activate native sequence NTNR α .

A “neutralizing antibody” is one which is able to block or significantly reduce an effector function of native sequence NTNR α . For example, a neutralizing antibody may inhibit or reduce NTNR α activation by a NTN ligand, as determined, for example, in a neurite survival assays, a NTN binding assay, or other assays taught herein or known in the art.

The phrase “enhancing proliferation of a cell” encompasses the step of increasing the extent of growth and/or reproduction of the cell relative to an untreated cell either in vitro or in vivo. An increase in cell proliferation in cell culture can be detected by counting the number of cells before and after exposure to a molecule of interest. The extent of proliferation can be quantified via microscopic examination of the degree of confluency. Cell proliferation can also be quantified using the thymidine incorporation assay described herein.

By “enhancing differentiation of a cell” is meant the act of increasing the extent of the acquisition or possession of one or more characteristics or functions which differ from that of the original cell (i.e. cell specialization). This can be detected by screening for a change in the phenotype of the cell (e.g., identifying morphological changes in the cell).

“Physiologically acceptable” carriers, excipients, or stabilizers are ones which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

As used herein, the term “salvage receptor binding epitope” refers to an epitope of the Fe region of an IgG molecule (e.g., IgG1, IgG2, IgG3, and IgG4) that is responsible for increasing the in vivo serum half-life of the IgG molecule. Exemplary salvage receptor binding epitope sequences include HQLNSDGK (SEQ ID NO:23); HQLNSDGK (SEQ ID NO:24); HQLSGTQ (SEQ ID NO:25); VISSHLGQ (SEQ ID NO:26); and PKNSSMISNTP (SEQ ID NO:27).

The term “cytokine” is a generic term for proteins released by one cell population which act on another cell as intercellular mediators. Examples of such cytokines are lymphokines, monokines, and traditional polypeptide hormones. Included among the cytokines are growth hormone such as human growth hormone, N-methionyl human growth hormone, and bovine growth hormone; parathyroid hormone; thyroxine; insulin; proinsulin; relaxin; prorelaxin; glycoprotein hormones such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), and luteinizing hormone (LH); hepatic growth factor; fibroblast growth factor; prolactin; placental lactogen; tumor necrosis factor- α and - β ; mullerian-inhibiting substance; mouse gonadotropin-associated peptide; inhibin; activin; vascular endothelial growth factor; integrin; thrombopoietin (TPO); neurotrophic factors or nerve growth factors such as NGF- β , NT-3, NT-4, NT-6, BDNF, CNTF, GDNF, AL-1 and other eph-receptor family ligands; platelet-growth factor; transforming growth factors (TGFs) such as TGF- α and TGF- β ; insulin-like growth factor-I and -II; erythropoietin (EPO); osteoinductive factors; interferons such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs) such as macrophage-CSF (M-CSF); granulocyte-macrophage-CSF (GM-CSF); and granulocyte-CSF (G-CSF); interleukins (ILs) such as IL-1, IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-11, IL-12; and other polypeptide factors kit ligand (KL). As used herein, the term cytokine includes proteins from natural sources or from recombinant cell culture and biologically active equivalents of the native sequence cytokines. Also included are genetically engineered molecules with cytokine activity such as TrkA-IgG or other soluble receptor chimeras.

“Treatment” refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

“Mammal” for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

By “solid phase” is meant a non-aqueous matrix to which a reagent of interest (e.g., the NTNR α or an antibody thereto) can adhere. Examples of solid phases encompassed herein include those formed partially or entirely of glass (e.g., controlled pore glass), polysaccharides (e.g., agarose), polyacrylamides, polystyrene, polyvinyl alcohol and silicones. In certain embodiments, depending on the context, the solid phase can comprise the well of an assay plate; in others it is a purification column (e.g., an affinity chromatography column). This term also includes a discontinuous solid phase of discrete particles, Such as those described in U.S. Pat. No. 4,275,149.

Modes for carrying out the invention are presented herein. Glial cell line-derived neurotrophic factor (“GDNF”) and Neurturin (“NTN”) are two structurally related, potent survival factors for sympathetic sensory and central nervous system neurons (Lin et al. *Science* 260:1130-1132 (1993);

Henderson et al. *Science* 266:1062–1064 (1994); Buj-Bello et al., *Neuron* 15:821–828 (1995); Kotzbauer et al. *Nature* 384:467–470 (1996)). Whereas GDNF was shown to mediate its actions through a multi-component receptor system composed of a ligand binding glycosyl-phosphatidyl inositol (GPI) linked protein (designated GDNFR α) and the transmembrane tyrosine kinase Ret (Treanor et al. *Nature* 382:80–83 (1996); Jing et al. *Cell* 85:1113–124 (1996); Trupp et al. *Nature* 381:785–789 (1996); Durbec et al. *Nature* 381:789–793 (1996)), the mechanism by which the NTN signal is transmitted has not been previously elucidated. Described herein is the isolation, sequence, and tissue distribution of a GPI-linked protein and its gene, designated NTN α , which is shown to modulate response to NTN but not GDNF. It is shown herein that it is structurally related to GDNFR α . Using recombinant proteins in a cell free system, it is shown that NTN α binds NTN (Kd~10 pM) but not GDNF, and that NTN does not bind GDNFR α with a high affinity. Also shown is that cellular responses to NTN require the presence of NTN α . Ligand bound NTN α induces phosphorylation of the tyrosine kinase receptor Ret. These findings identify Ret and NTN α , respectively, as signalling and ligand binding components of a receptor for NTN and related ligands. This defines a novel neurotrophic and differentiation factor receptor family of receptors containing a shared transmembrane protein tyrosine kinase (Ret) and a ligand specific GPI-linked protein (NTN α).

Glial cell line-derived neurotrophic factor (“GDNF”) (Lin et al., *Science*, 260:1130–1132 (1993); WO 93/06116, which are incorporated herein in its entirety), is a potent survival factor for midbrain dopaminergic (Lin et al., *Science*, 260:1130–1132 (1993); Strömberg et al., *Exp. Neurol.*, 124:401–412 (1993); Beck et al., *Nature*, 373:339–341 (1995); Kearns et al. *Brain Res.*, 672:104–111 (1995); Tomac et al., *Nature*, 373:335–339 (1995)) spinal motor (Henderson et al., *Science*, 266:1062–1064 (1994); Oppenheim et al., *Nature*, 373:344–346 (1995); Yan et al., *Nature*, 373:341–344 (1995)) and noradrenergic neurons (Arenas et al., *Neuron*, 15:1465–1473 (1995)), which degenerate in Parkinson’s disease (Hirsch et al., *Nature*, 334:345–348 (1988); Hornykiewicz *Mt. Sinai J. Med.*, 55:11–20 (1988)), amyotrophic lateral sclerosis (Hirano, *Amyotrophic Lateral Sclerosis and Other Motor Neuron Diseases*, P. Rowland, ed. (New York: Raven Press, Inc.) pp. 91–101 (1991)), and Alzheimer’s disease (Marcyniuk et al., *J. Neurol. Sci.*, 76:335–345 (1986); Cash et al., *Neurology*, 37:42–46 (1987); Chan-Palay et al., *Comp. Neurol.*, 287:373–392 (1989)) respectively. Based on mice genetically engineered to lack GDNF, additional biological roles for GDNF have been reported: the development and/or survival of enteric, sympathetic, and sensory neurons and the renal system, but not for catecholaminergic neurons in the central nervous system (CNS) (Moore et al. *Nature* 382:76–79 (1996); Pichel et al. *Nature* 382:73–76 (1996); Sanchez et al. *Nature* 382:70–73 (1996)). Despite the physiological and clinical importance of GDNF, little is known about its mechanism of action.

Cytokine receptors frequently assemble into multi-subunit complexes. Sometimes, the α subunit of this complex is involved in binding the cognate growth factor and the β -subunit may contain an ability to transduce a signal to the cell. Without wishing to be bound by theory, these receptors have been assigned to three subfamilies depending on the complexes formed. Subfamily 1 includes the receptors for EPO, granulocyte colony-stimulating factor (G-CSF), interleukin-4 (IL-4), interleukin-7 (IL-7), growth hormone (GH), and prolactin (PRL). Ligand binding to receptors

belonging to this subfamily is thought to result in homodimerization of the receptor. Subfamily 2 includes receptors for IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-5 (IL-5), interleukin-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF). Subfamily 2 receptors are heterodimers having an α -subunit for ligand binding, and β -subunit (either the shared β -subunit of the IL-3, GM-CSF, and IL-5 receptors or the gp130 subunit of the IL-6, LIF, OSM, and CNTF receptors) for signal transduction. Subfamily 3 contains only the interleukin-2 (IL-2) receptor. The β and γ subunits of the IL-2 receptor complex are cytokine-receptor polypeptides which associate with the α -subunit of the unrelated Tac antigen.

The present invention is based on the discovery of the NTN α , a protein that binds NTN with a high affinity. The experiments described herein demonstrate that this molecule is a receptor which appears to play a role in mediating responses to NTN. In particular, this receptor has been found to be present in a variety of tissue and cell populations, including neurons, thus indicating that NTN ligands, such as agonist antibodies, can be used to stimulate proliferation, growth, survival, differentiation, metabolism, or regeneration of NTN α - and Ret-containing cells.

Techniques suitable for the production of NTN α are well known in the art and include isolating NTN α from an endogenous source of the polypeptide, peptide synthesis (using a peptide synthesizer) and recombinant techniques (or any combination of these techniques). The preferred technique for production of NTN α is a recombinant technique to be described below.

Most of the discussion below pertains to recombinant production of NTN α by culturing cells transformed with a vector containing NTN α nucleic acid and recovering the polypeptide from the cell culture. It is further envisioned that the NTN α of this invention may be produced by homologous recombination, as provided for in WO 91/06667, published May 16, 1991.

Briefly, this method involves transforming primary human cells containing a NTN α -encoding gene with a construct (i.e., vector) comprising an amplifiable gene (such as dihydrofolate reductase (DHFR) or others discussed below) and at least one flanking region of a length of at least about 150 bp that is homologous with a DNA sequence at the locus of the coding region of the NTN α gene to provide amplification of the NTN α gene. The amplifiable gene must be at a site that does not interfere with expression of the NTN α gene. The transformation is conducted such that the construct becomes homologously integrated into the genome of the primary cells to define an amplifiable region.

Primary cells comprising the construct are then selected for by means of the amplifiable gene or other marker present in the construct. The presence of the marker gene establishes the presence and integration of the construct into the host genome. No further selection of the primary cells need be made, since selection will be made in the second host. If desired, the occurrence of the homologous recombination event can be determined by employing PCR and either sequencing the resulting amplified DNA sequences or determining the appropriate length of the PCR fragment when DNA from correct homologous integrants is present and expanding only those cells containing such fragments. Also if desired, the selected cells may be amplified at this point by stressing the cells with the appropriate amplifying agent (such as methotrexate if the amplifiable gene is DHFR), so

that multiple copies of the target gene are obtained. Preferably, however, the amplification step is not conducted until after the second transformation described below.

After the selection step, DNA portions of the genome, sufficiently large to include the entire amplifiable region, are isolated from the selected primary cells. Secondary mammalian expression host cells are then transformed with these genomic DNA portions and cloned, and clones are selected that contain the amplifiable region. The amplifiable region is then amplified by means of an amplifying agent if not already amplified in the primary cells. Finally, the secondary expression host cells now comprising multiple copies of the amplifiable region containing NTNR α are grown so as to express the gene and produce the protein.

The conserved structure and sequence of the mammalian NTNR α and the elucidation of the cDNA sequence which encodes the rat and mouse receptor, as well as human sequences disclosed herein, make it possible to clone gene sequences from other mammals which encode the NTNR α . Of particular interest to the present invention is the ability to clone the human NTNR α molecules using the sequences disclosed herein. The DNA encoding NTNR α may be obtained from any cDNA library prepared from tissue believed to possess the NTR α mRNA and to express it at a detectable level, as shown herein in the Examples. Accordingly, NTNR α DNA can be conveniently obtained from a cDNA library prepared, for example, from mammalian fetal liver, brain, muscle, intestine, and peripheral nerves. The NTNR α -encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries are screened with probes (such as antibodies to the NTNR α or oligonucleotides of about 20–80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures as described in chapters 10–12 of Sambrook et al., *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding NTNR α is to use PCR methodology as described in section 14 of Sambrook et al., *supra*.

A preferred method of practicing this invention is to use carefully selected oligonucleotide sequences to screen cDNA libraries from various human tissues, preferably human fetal liver. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. Preferred sequences are obtained from the naturally-occurring NTNR α disclosed herein.

The oligonucleotide must be labeled such that it can be detected upon hybridization to DNA in the library being screened. The preferred method of labeling is to use ³²P-labeled ATP with polynucleotide kinase, as is well known in the art, to radiolabel the oligonucleotide. However, other methods may be used to label the oligonucleotide, including, but not limited to, biotinylation or enzyme labeling.

Amino acid sequence variants of NTNR α are prepared by introducing appropriate nucleotide changes into the NTNR α DNA, or by synthesis of the desired NTNR α polypeptide. Such variants represent insertions, substitutions, and/or specified deletions of, residues within or at one or both of the ends of the amino acid sequence of a naturally occurring NTNR α , such as the NTNR α shown in FIGS. 1A–1C or 2A–2D or sequences disclosed herein. Preferably, these variants represent insertions and/or substitutions within or at one or both ends of the mature sequence, and/or insertions,

substitutions and/or specified deletions within or at one or both of the ends of the signal sequence of the NTNR α . Any combination of insertion, substitution, and/or specified deletion is made to arrive at the final construct, provided that the final construct possesses the desired biological activity as defined herein. The amino acid changes also may alter post-translational processes of the NTNR α , such as changing the number or position of glycosylation sites, altering the membrane anchoring characteristics, and/or altering the intracellular location of the NTNR α by inserting, deleting, or otherwise affecting the leader sequence of the NTNR α .

Variations in the native sequence as described above can be made using any of the techniques and guidelines for conservative and non-conservative mutations set forth in U.S. Pat. No. 5,364,934. These include oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. See also, for example, Table I therein and the discussion surrounding this table for guidance on selecting amino acids to change, add, or delete.

The nucleic acid (e.g., cDNA or genomic DNA) encoding the NTNR α is inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Many vectors are available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

The NTNR α s of this invention may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the NTNR α DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native NTNR α signal sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, 1 pp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, e.g., the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders, the latter described in U.S. Pat. No. 5,010, 182 issued Apr. 23, 1991), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published Apr. 4, 1990), or the signal described in WO 90/13646 published Nov. 15, 1990. In mammalian cell expression the native signal sequence (e.g., the NTNR α presequence that normally directs secretion of NTNR α from human cells in vivo) is satisfactory, although other mammalian signal sequences may be suitable, such as signal sequences from other animal NTNR α s, and signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

The DNA for such precursor region is ligated in reading frame to DNA encoding the mature NTNR α or a soluble variant thereof.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such

sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of NTNR α DNA. However, the recovery of genomic DNA encoding NTNR α is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the NTNR α DNA.

Expression and cloning vectors should contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the NTNR α nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes NTNR α . Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of NTNR α are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc. A preferred vector system is provided in U.S. Pat. No. 5,561,053.

For example, cells transformed with the DHFR selection gene are first identified by culturing all of the transformants

in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding NTNR α . This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding NTNR α , wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Pat. No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb et al., *Nature*, 282:39 (1979)). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20.622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts. Bianchi et al., *Curr. Genet.*, 12:185 (1987). More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer et al., *Bio/Technology*, 9:968-975(1991).

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the NTNR α nucleic acid. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the NTNR α nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to NTNR α -encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native NTNR α promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the NTNR α DNA. However, heterologous promoters are

preferred, as they generally permit greater transcription and higher yields of NTN α as compared to the native NTN α promoter.

Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems (Chang et al., *Nature*, 275:615 (1978); Goeddel et al., *Nature*, 281:544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, *Nucleic Acids Res.*, 8:4057 (1980); EP 36,776), and hybrid promoters such as the tac promoter. deBoer et al., *Proc. Natl. Acad. Sci. USA*, 80:21-25 (1983). However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding NTN α (Siebenlist et al., *Cell*, 20:269 (1980)) using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Delgarno (S.D.) sequence operably linked to the DNA encoding NTN α .

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem.*, 255:2073 (1980)) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg.*, 7:149 (1968); Holland, *Biochemistry*, 17:4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

NTN α transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published Jul. 5, 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the NTN α sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. Fiers et al., *Nature*, 273:113 (1978); Mulligan et al., *Science*, 209:1422-1427 (1980); Pavlakis et al., *Proc. Natl. Acad.*

Sci. USA, 78:7398-7402 (1981). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. Greenaway et al., *Gene*, 18:355-360 (1982). A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Pat. No. 4,419,446. A modification of this system is described in U.S. Pat. No. 4,601,978. See also Gray et al., *Nature*, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., *Nature*, 297:598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani et al., *Proc. Natl. Acad. Sci. USA*, 79:5166-5170 (1982) on expression of the human interferon β 1 gene in cultured mouse and rabbit cells; and Gorman et al., *Proc. Natl. Acad. Sci. USA*, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter.

Transcription of a DNA encoding the NTN α of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' (Laimins et al., *Proc. Natl. Acad. Sci. USA*, 78:993(1981)) and 3' (Lusky et al., *Mol. Cell Bio.*, 3:1108(1983)) to the transcription unit, within an intron (Banerji et al., *Cell*, 33:729 (1983)), as well as within the coding sequence itself. Osborne et al., *Mol. Cell Bio.*, 4:1293 (1984). Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature*, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the NTN α -encoding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding NTN α .

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures are used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., *Nucleic Acids Res.*, 9:309 (1981) or by the method of Maxam et al., *Methods in Enzymology*, 65:499 (1980).

Particularly useful in the practice of this invention are expression vectors that provide for the transient expression in mammalian cells of DNA encoding NTNR α . In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector. Sam brook et al., supra, pp. 16.17–16.22. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying analogs and variants of NTNR α that are biologically active NTNR α .

Other methods, vectors, and host cells suitable for adaptation to the synthesis of NTNR α in recombinant vertebrate cell culture are described in Gething et al., *Nature*, 293:620–625 (1981); Mantei et al., *Nature*, 281:40–46 (1979); EP 117,060; and EP 117,058. A particularly useful plasmid for mammalian cell culture expression of NTNR α is pRK5 (EP 307,247) or pSV16B. WO 91/08291 published Jun. 13, 1991.

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as Bacilli such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published Apr. 12, 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting. Strain W3110 is a particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in the genes encoding proteins, with examples of such hosts including *E. coli* W3110 strain 27C7. The complete genotype of 27C7 is tonA Δ ptr3 phoA Δ E15 Δ (argF-lac)169 ompT Δ degP41kan^r. Strain 27C7 was deposited on Oct. 30, 1991 in the American Type Culture Collection as ATCC No. 55,244. Alternatively, the strain of *E. coli* having mutant periplasmic protease disclosed in U.S. Pat. No. 4,946,783 issued Aug. 7, 1990 may be employed. Alternatively still, methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for NTNR α -encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe* (Beach et al., *Nature*, 290:140 (1981); EP 139,383 published May 2, 1985); *Kluyveromyces* hosts (U.S. Pat. No. 4,943,529; Fleer et al., supra) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt et al., *J. Bacteriol.*, 737 (1983)), *K. fragilis* (ATCC 12,424), *K.*

bulgaricus (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilorum* (ATCC 36,906; Van den Berg et al., supra), *K. thermotolerans*, and *K. marxianus*; *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishnia et al., *J. Basic Microbiol.*, 28:265–278 (1988)); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case et al., *Proc. Natl. Acad. Sci. USA*, 76:5259–5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published Oct. 31, 1990); and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium* (WO 91/00357 published Jan. 10, 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., *Biochem. Biophys. Res. Commun.*, 112:284–289 (1983); Tilburn et al., *Gene*, 26:205–221 (1983); Yelton et al., *Proc. Natl. Acad. Sci. USA*, 81:1470–1474 (1984)) and *A. niger*. Kelly et al., *EMBO J.*, 4:475–479 (1985).

Suitable host cells for the expression of glycosylated NTNR α are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. See, e.g., Luckow et al., *Bio/Technology*, 6:47–55 (1988); Miller et al., in *Genetic Engineering*, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277–279; and Maeda et al., *Nature*, 315:592–594 (1985). A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bin-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*, which has been previously manipulated to contain the NTNR α -encoding DNA. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the NTNR α is transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the NTNR α -encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences. Depicker et al., *J. Mol. Appl. Gen.*, 1:561 (1982). In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue. EP 321,196 published Jun. 21, 1989.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure. See, e.g., *Tissue Culture*, Academic Press, Kruse and Patterson, editors (1973). Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., *J. Gen. Virol.*, 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub et al., *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.*,

23:243–251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., *Annals N.Y. Acad. Sci.*, 383:44–68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for NTN α production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO₄ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in section 1.82 of Sam brook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., *Gene*, 23:315 (1983) and WO 89/05859 published Jun. 29, 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published Jan. 10, 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham et al., *Virology*, 52:456–457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216 issued Aug. 16, 1983. Transformations into yeast are typically carried out according to the method of Van Solingen et al., *J. Bact.*, 130:946 (1977) and Hsiao et al., *Proc. Natl. Acad. Sci. USA*, 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, etc., may also be used. For various techniques for transforming mammalian cells, see Keown et al., *Methods in Enzymology*, 185:527–537 (1990) and Mansour et al., *Nature*, 336:348–352 (1988).

Prokaryotic cells used to produce the NTN α polypeptide of this invention are cultured in suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce the NTN α of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham et al. *Meth. Enz.*, 58:44(1979), Barnes et al., *Anal. Biochem.*, 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Pat. Re. 30,985 may be used as culture media for the

host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in *Mammalian Cell Biotechnology: a Practical Approach*, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, *Proc. Natl. Acad. Sci. USA*, 77:5201–5205 (1980)), dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, particularly ³²P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionuclides, fluorescers, enzymes, or the like. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, can be measured by immunological methods, such as immunohistochemical staining of tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, luminescent labels, and the like. A particularly sensitive staining technique suitable for use in the present invention is described by Hsu et al., *Am. J. Clin. Path.*, 75:734–738 (1980).

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared as described herein.

NTN α (e.g., NTN α ECD) preferably is recovered from the culture medium as a secreted polypeptide, although it also may be recovered from host cell lysates. If the NTN α is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100).

When NTN α is produced in a recombinant cell other than one of human origin, the NTN α is completely free of

proteins or polypeptides of human origin. However, it is necessary to purify NTN α from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to NTN α . As a first step, the culture medium or lysate can be centrifuged to remove particulate cell debris. NTN α can then be purified from contaminants soluble proteins and polypeptides with the following procedures, which are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica; chromatofocusing; immunoaffinity; epitope-tag binding resin; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

NTN α variants in which residues have been deleted, inserted, or substituted are recovered in the same fashion as native sequence NTN α , taking account of any substantial changes in properties occasioned by the variation. Immunoaffinity resins, such as a monoclonal anti-NTN α resin, can be employed to absorb the NTN α variant by binding it to at least one remaining epitope.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants.

Covalent modifications of NTN α polypeptides are included within the scope of this invention. Both native sequence NTN α and amino acid sequence variants of the NTN α may be covalently modified. One type of covalent modification of the NTN α is introduced into the molecule by reacting targeted amino acid residues of the NTN α with an organic derivatizing agent that is capable of reacting the N-terminal residue, the C-terminal residue, or with selected side chains.

Cysteinyl residues most commonly are reacted with α -haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α -bromo- β -(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylpyrocarbonate at pH 5.5–7.0 because this agent is relatively specific for the histidyl side chain. Parabromophenacyl bromide also is useful; the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α -amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed under alkaline conditions because of the high pK_a of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as with the arginine epsilon-amino group.

The specific modification of tyrosyl residues may be made, with particular interest in introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-acetylimidazole and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively. Tyrosyl residues are iodinated using ¹²⁵I or ¹³¹I to prepare labeled proteins for use in radioimmunoassay, the chloramine T method being suitable.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N=C=N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Derivatization with bifunctional agents is useful for crosslinking NTN α to a water-insoluble support matrix or surface for use in the method for purifying anti-NTN α antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-((p-azidophenyl)dithio)propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Pat. Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Glutaminyl and asparaginyl residues are frequently deamidated to the corresponding glutamyl and aspartyl residues, respectively. These residues are deamidated under neutral or basic conditions. The deamidated form of these residues falls within the scope of this invention.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains (T. E. Creighton, *Proteins: Structure and Molecular Properties*, W. H. Freeman & Co., San Francisco, pp. 79–86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the NTN α polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. By altering is meant deleting one or more carbohydrate moieties found in native NTN α , and/or adding one or more glycosylation sites that are not present in the native NTN α .

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to

a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the NTN α polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native NTN α sequence (for O-linked glycosylation sites). For ease, the NTN α amino acid sequence is preferably altered through changes at the DNA level, particularly by mutating the DNA encoding the NTN α polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the NTN α polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. These procedures are advantageous in that they do not require production of the polypeptide in a host cell that has glycosylation capabilities for N- or O-linked glycosylation. Depending on the coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulphydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 87/05330 published Sep. 11, 1987, and in Aplin et al., *CRC Crit. Rev. Biochem.*, 259-306 (1981).

Removal of carbohydrate moieties present on the NTN α polypeptide may be accomplished chemically or enzymatically. Chemical deglycosylation requires exposure of the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound. This treatment results in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., *Arch. Biochem. Biophys.*, 259:52 (1987) and by Edge et al., *Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., *Meth. Enzymol.*, 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., *J. Biol. Chem.*, 257:3105(1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of NTN α comprises linking the NTN α polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Pat. Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Variants can be assayed as taught herein. A change in the immunological character of the NTN α molecule, such as affinity for a given antibody, can be measured by a competitive-type immunoassay. Other potential modifications of protein or polypeptide properties such as redox or thermal stability, hydrophobicity, susceptibility to proteolytic degradation, or the tendency to aggregate with carriers or into multimers are assayed by methods well known in the art.

This invention encompasses chimeric polypeptides comprising NTN α fused to a heterologous polypeptide. A chimeric NTN α is one type of NTN α variant as defined herein. In one preferred embodiment, the chimeric polypeptide comprises a fusion of the NTN α with a tag polypeptide which provides an epitope to which an anti-tag antibody or molecule can selectively bind. The epitope-tag is generally provided at the amino- or carboxyl-terminus of the NTN α . Such epitope-tagged forms of the NTN α are desirable, as the presence thereof can be detected using a labeled antibody against the tag polypeptide. Also, provision of the epitope tag enables the NTN α to be readily purified by affinity purification using the anti-tag antibody. Affinity purification techniques and diagnostic assays involving antibodies are described later herein.

Tag polypeptides and their respective antibodies are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 (Field et al., *Mol. Cell. Biol.*, 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., *Molecular and Cellular Biology*, 5:3610-3616 (1985)); and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody. Paborsky et al., *Protein Engineering*, 3(6):547-553 (1990). Other tag polypeptides have been disclosed. Examples include the Flag-peptide (Hopp et al., *BioTechnology*, 6:1204-1210 (1988)); the KT3 epitope peptide (Martin et al., *Science*, 255:192-194 (1992)); an α -tubulin epitope peptide (Skinner et al., *J. Biol. Chem.*, 266:15163-15166 (1991)); and the T7 gene 10 protein peptide tag. Lutz-Freyermuth et al., *Proc. Natl. Acad. Sci. USA*, 87:6393-6397(1990). Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein. A C-terminal poly-histidine sequence tag is preferred. Poly-histidine sequences allow isolation of the tagged protein by Ni-NTA chromatography as described (Lindsay et al. *Neuron* 17:571-574 (1996)), for example.

The general methods suitable for the construction and production of epitope-tagged NTN α are the same as those disclosed hereinabove. NTN α -tag polypeptide fusions are most conveniently constructed by fusing the cDNA sequence encoding the NTN α portion in-frame to the tag polypeptide DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the NTN α -tag polypeptide chimeras of the present invention, nucleic acid encoding the NTN α will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions are also possible.

Epitope-tagged NTN α can be conveniently purified by affinity chromatography using the anti-tag antibody. The matrix to which the affinity antibody is attached is most often agarose, but other matrices are available (e.g. controlled pore glass or poly(styrenedivinyl)benzene). The epitope-tagged NTN α can be eluted from the affinity column by varying the buffer pH or ionic strength or adding chaotropic agents, for example.

Chimeras constructed from a receptor sequence linked to an appropriate immunoglobulin constant domain sequence (immunoadhesins) are known in the art. Immunoadhesins reported in the literature include fusions of the T cell receptor* (Gascoignecet al., *Proc. Natl. Acad. Sci. USA*, 84:2936-2940 (1987)); CD4* (Capon et al., *Nature* 337:525-531 (1989); Trauneker et al., *Nature*, 339:68-70 (1989); Zettmeissl et al., *DNA Cell Biol. USA*, 9:347-353 (1990); Byrn et al., *Nature*, 344:667-670 (1990)); L-selectin (homing receptor) ((Watson et al., *J. Cell. Biol.*, 110:2221-2229 (1990); Watson et al., *Nature*, 349: 164-167

(1991)); CD44* (Aruffo et al., *Cell*, 61:1303–1313 (1990)); CD28* and B7* (Linsley et al., *J. Exp. Med.*, 173:721–730 (1991)); CTLA-4* (Lisley et al., *J. Exp. Med.* 174:561–569 (1991)); CD22* (Stamenkovic et al., *Cell*, 66:1133–1144 (1991)); TNF receptor (Ashkenazi et al., *Proc. Natl. Acad. Sci. USA*, 88:10535–10539 (1991); Lesslauer et al., *Eur. J. Immunol.*, 27: 2883–2886 (1991); Peppel et al., *J. Exp. Med.*, 174:1483–1489 (1991)); NP receptors (Bennett et al., *J. Biol. Chem.* 266:23060–23067(1991)); and IgE receptor α^* (Ridgway et al., *J. Cell. Biol.*, 115:abstr. 1448 (1991)), where the asterisk (*) indicates that the receptor is member of the immunoglobulin superfamily.

The simplest and most straightforward immunoadhesin design combines the binding region(s) of the “adhesin” protein with the hinge and Fe regions of an immunoglobulin heavy chain. Ordinarily, when preparing the NTNR α -immunoglobulin chimeras of the present invention, nucleic acid encoding the extracellular domain of the NTNR α will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge and CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the NTNR α -immunoglobulin chimeras.

In some embodiments, the NTNR α -immunoglobulin chimeras are assembled as monomers, or hetero- or homomultimer, and particularly as dimers or tetramers, essentially as illustrated in WO 91/08298.

In a preferred embodiment, the NTNR α extracellular domain sequence is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. Immunoglobulin G₁ (IgG1). It is possible to fuse the entire heavy chain constant region to the NTNR α extracellular domain sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114, or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the NTNR α amino acid sequence is fused to the hinge region and CH2 and CH3, or to the CH1, hinge, CH2 and CH3 domains of an IgG1, IgG2, or IgG3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

In some embodiments, the NTNR α -immunoglobulin chimeras are assembled as multimer, and particularly as homodimers or -tetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each four unit may be the same or different.

Alternatively, the NTNR α extracellular domain sequence can be inserted between immunoglobulin heavy chain and

light chain sequences such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment the NTNR α sequence is fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the CH2 domain, or between the CH2 and CH3 domains. Similar constructs have been reported by Hoogenboom et al., *Mol. Immunol.*, 28:1027–1037(1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an NTNR α -immunoglobulin heavy chain fusion polypeptide, or directly fused to the NTNR α extracellular domain. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the NTNR α -immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567 issued Mar. 28, 1989.

In a preferred embodiment, the immunoglobulin sequences used in the construction of the immunoadhesins of the present invention are from an IgG immunoglobulin heavy chain constant domain. For human immunoadhesins, the use of human IgG1 and IgG3 immunoglobulin sequences is preferred. A major advantage of using IgG1 is that IgG1 immunoadhesins can be purified efficiently on immobilized protein A. In contrast, purification of IgG3 requires protein G, a significantly less versatile medium. However, other structural and functional properties of immunoglobulins should be considered when choosing the Ig fusion partner for a particular immunoadhesin construction. For example, the IgG3 hinge is longer and more flexible, so it can accommodate larger adhesin domains that may not fold or function properly when fused to IgG1. Another consideration may be valency; IgG immunoadhesins are bivalent homodimers, whereas Ig subtypes like IgA and IgM may give rise to dimeric or pentameric structures, respectively, of the basic Ig homodimer unit. For NTNR α immunoadhesins designed for in vivo application, the pharmacokinetic properties and the effector functions specified by the Fc region are important as well. Although IgG1, IgG2 and IgG4 all have in vivo half-lives of 21 days, their relative potencies at activating the complement system are different. IgG4 does not activate complement, and IgG2 is significantly weaker at complement activation than IgG1. Moreover, unlike IgG 1, IgG2 does not bind to Fc receptors on mononuclear cells or neutrophils. While IgG3 is optimal for complement activation, its in vivo half-life is approximately one third of the other IgG isotypes. Another important consideration for immunoadhesins designed to be used as human therapeutics is the number of allotypic variants of the particular isotype. In general, IgG isotypes with fewer serologically-defined allotypes are preferred. For example, IgG1 has only four serologically-defined allotypic sites, two of which (G1m and 2) are located in the Fe region; and one of these sites G1ml, is non-immunogenic. In contrast, there are 12 serologically-defined allotypes in IgG3, all of which are in the Fc region; only three of these sites (G3m5, 11 and 21) have one allotype which is nonimmunogenic. Thus, the potential immunogenicity of a γ 3 immunoadhesin is greater than that of a γ 1 immunoadhesin.

With respect to the parental immunoglobulin, a useful joining point is just upstream of the cysteines of the hinge

that form the disulfide bonds between the two heavy chains. In a frequently used design, the codon for the C-terminal residue of the NTN α part of the molecule is placed directly upstream of the codons for the sequence DKTHTCPPCP (SEQ ID NO:28 of the IgG1 hinge region).

The general methods suitable for the construction and expression of immunoadhesins are the same as those disclosed hereinabove with regard to NTN α . NTN α immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the NTN α portion in-frame to an Ig cDNA sequence. However, fusion to genomic Ig fragments can also be used (see, e.g., Gascoigne et al., *Proc. Natl. Acad. Sci. USA*, 84:2936–2940 (1987); Aruffo et al., *Cell*, 61:1303–1313 (1990); Stamenkovic et al., *Cell*, 66:1133–1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequence from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the NTN α and Ig parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells. For expression in mammalian cells, pRK5-based vectors (Schall et al., *Cell*, 61:361–370 (1990)) and CDM8-based vectors (Seed, *Nature*, 329:840 (1989)) can be used. The exact junction can be created by removing the extra sequences between the designed junction codons using oligonucleotide-directed deletional mutagenesis (Zoller et al., *Nucleic Acids Res.*, 10:6487 (1982); Capon et al., *Nature*, 337:525–531 (1989)). Synthetic oligonucleotides can be used, in which each half is complementary to the sequence on either side of the desired junction; ideally, these are 36 to 48-mers. Alternatively, PCR techniques can be used to join the two parts of the molecule in-frame with an appropriate vector.

The choice of host cell line for the expression of NTN α immunoadhesins depends mainly on the expression vector. Another consideration is the amount of protein that is required. Milligram quantities often can be produced by transient transfections. For example, the adenovirus EIA-transformed 293 human embryonic kidney cell line can be transfected transiently with pRK5-based vectors by a modification of the calcium phosphate method to allow efficient immunoadhesin expression. CDM8-based vectors can be used to transfect COS cells by the DEAE-dextran method (Aruffo et al., *Cell*, 61:1303–1313 (1990); Zettmeissl et al., *DNA Cell Biol. US*, 9:347–353 (1990)). If larger amounts of protein are desired, the immunoadhesin can be expressed after stable transfection of a host cell line. For example, a pRK5-based vector can be introduced into Chinese hamster ovary (CHO) cells in the presence of an additional plasmid encoding dihydrofolate reductase (DHFR) and conferring resistance to G418. Clones resistant to G418 can be selected in culture; these clones are grown in the presence of increasing levels of DHFR inhibitor methotrexate; clones are selected, in which the number of gene copies encoding the DHFR and immunoadhesin sequences is co-amplified. If the immunoadhesin contains a hydrophobic leader sequence at its N-terminus, it is likely to be processed and secreted by the transfected cells. The expression of immunoadhesins with more complex structures may require uniquely suited host cells; for example, components such as light chain or J chain may be provided by certain myeloma or hybridoma cell hosts (Gascoigne et al., 1987, supra, Martin et al., *J. Virol.*, 67:3561–3568 (1993)).

Immunoadhesins can be conveniently purified by affinity chromatography. The suitability of protein A as an affinity

ligand depends on the species and isotype of the immunoglobulin Fc domain that is used in the chimera. Protein A can be used to purify immunoadhesins that are based on human γ 1, γ 2, or γ 4 heavy chains (Lindmark et al., *J. Immunol. Meth.*, 62:1–13 (1983)). Protein G is recommended for all mouse isotypes and for human γ 3 (Guss et al., *EMBO J.*, 5:1567–1575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrene-divinyl)benzene allow for faster flow rates and shorter processing times than can be achieved with agarose. The conditions for binding an immunoadhesin to the protein A or G affinity column are dictated entirely by the characteristics of the Fc domain; that is, its species and isotype. Generally, when the proper ligand is chosen, efficient binding occurs directly from unconditioned culture fluid. One distinguishing feature of immunoadhesins is that, for human γ 1 molecules, the binding capacity for protein A is somewhat diminished relative to an antibody of the same Fc type. Bound immunoadhesin can be efficiently eluted either at acidic pH (at or above 3.0), or in a neutral pH buffer containing a mildly chaotropic salt. This affinity chromatography step can result in an immunoadhesin preparation that is >95% pure.

Other methods known in the art can be used in place of, or in addition to, affinity chromatography on protein A or G to purify immunoadhesins. Immunoadhesins behave similarly to antibodies in thiophilic gel chromatography (Hutchens et al., *Anal. Biochem.*, 159:217–226 (1986)) and immobilized metal chelate chromatography (Al-Mashikhi et al., *J. Dairy Sci.*, 71:1756–1763 (1988)). In contrast to antibodies, however, their behavior on ion exchange columns is dictated not only by their isoelectric points, but also by a charge dipole that may exist in the molecules due to their chimeric nature.

If desired, the immunoadhesin can be made bispecific. Thus, the immunoadhesins of the present invention may combine a NTN α extracellular domain and a domain, such as the extracellular domain, of another cytokine or neurotrophic factor receptor subunit. Exemplary cytokine receptors from which such bispecific immunoadhesin molecules can be made include TPO (or mpl ligand), EPO, G-CSF, IL-4, IL-7, GH, PRL, IL-3, GM-CSF, IL-5, IL-6, LIF, OSM, CNTF, GDNF and IL-2 receptors. For bispecific molecules, trimeric molecules, composed of a chimeric antibody heavy chain in one arm and a chimeric antibody heavy chain-light chain pair in the other arm of their antibody-like structure are advantageous, due to ease of purification. In contrast to antibody-producing quadromas traditionally used for the production of bispecific immunoadhesins, which produce a mixture of ten tetramers, cells transfected with nucleic acid encoding the three chains of a trimeric immunoadhesin structure produce a mixture of only three molecules, and purification of the desired product from this mixture is correspondingly easier.

The NTN α protein and NTN α gene are believed to find ex vivo or in vivo therapeutic use for administration to a mammal, particularly humans, in the treatment of diseases or disorders, related to neurturin activity or benefited by neurturin-responsiveness. See Kotzbauer et al. *Nature* 384:467–470 (1996), which is specifically incorporated herein by reference. Conditions particularly amenable to treatment with the embodiments of the invention are those related to Ret expression or that benefit by Ret activation, particularly of the downstream pathways mediated by Ret. See Treanor et al. *Nature* 382:80–83 (1996); Jing et al. *Cell* 85:1113–1124 (1996); Trupp et al. *Nature* 381:785–789

(1996); and Durbec et al. *Nature* 381:789–793 (1996), which are specifically incorporated herein by reference. Particularly preferred are neurologic disorders, preferably central nervous system disorders, disorders of the kidney, hematopoietic disorders related to the spleen, and enteric nervous system disorders. The patient is administered an effective amount of NTNR α protein, peptide fragment, or variant of the invention. Therapeutic methods comprising administering NTNR α , NTNR α agonists, NTNR α antagonists (which compete with endogenous NTN), or anti-NTNR α antibodies are within the scope of the present invention. The present invention also provides for pharmaceutical compositions comprising NTNR α protein, peptide fragment, or derivative in a suitable pharmacologic carrier. The NTNR α protein, peptide fragment, or variant may be administered systemically or locally. Applicable to the methods taught herein, the receptor protein can be optionally administered prior to, after, or preferably concomitantly with (or in complex with) NTN or other NTNR α ligand. As taught herein, NTNR α can be provided to target cells in the absence of NTN to increase the responsiveness of those cells to subsequently administered NTN or NTN agonist.

It may be beneficial to decrease the trophic effect of endogenous NTN. Therefore, in areas of nervous system trauma, it may be desirable to provide NTN antagonists, including, but not limited to, cell-free NTNR α , which may compete with endogenous cellular receptor for NTN binding. Under such circumstances, it may be desirable to provide NTN antagonist locally at the injury site rather than systemically. Use of a NTNR providing implant may be desirable.

Alternatively, certain conditions can benefit from an increase in NTN (or other NTNR α -ligand) responsiveness. It may therefore be beneficial to increase the number of or binding affinity of NTNR α in cells of patients suffering from such conditions. This can be achieved through administration of soluble NTNR α , optionally complexed with NTNR α -ligand, preferably NUN, or by gene therapy using NTNR α -encoding nucleic acid. Selective expression of recombinant NTNR in appropriate cells could be achieved using NTNR genes controlled by tissue specific or inducible promoters or by producing localized infection with replication defective viruses carrying a recombinant NTNR gene. Conditions which may benefit from increased sensitivity to NTN include, but are not limited to, motoneuron disorders including amyotrophic lateral sclerosis, Werdnig-Hoffmann disease, chronic proximal spinal muscular atrophy, and Guillain-Barre syndrome. Additional conditions include those involving sympathetic neurons, particularly where increased survival or NTN-responsiveness is desired. Conditions where increased survival or NTN-responsiveness of sensory neurons, including peripheral sensory neurons, and central nervous system neurons, including dopaminergic neurons, is desirable are also suitably treated with embodiments of the invention. Accordingly, treatment of neurological disorders associated with diabetes, Parkinson's disease, Alzheimer's disease, and Huntington's chorea are provided herein. The present methods can also be applied to conditions related to non-neuronal cells that express NTNR α . In fact, since NTNR α serves to activate Ret, conditions associated with Ret-expressing cells can be treated with the embodiments of the invention.

A disease or medical disorder is considered to be nerve damage if the survival or function of nerve cells and/or their axonal processes is compromised. Such nerve damage occurs as the result conditions including (a) Physical injury, which causes the degeneration of the axonal processes

and/or nerve cell bodies near the site of the injury; (b) Ischemia, as a stroke; (c) Exposure to neurotoxins, such as the cancer and AIDS chemotherapeutic agents such as cisplatin and dideoxycytidine (ddC), respectively; (d) Chronic metabolic diseases, such as diabetes or renal dysfunction; and (e) Neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis (ALS), which cause the degeneration of specific neuronal populations. Conditions involving nerve damage include Parkinson's disease, Alzheimer's disease, Amyotrophic Lateral Sclerosis, stroke, diabetic polyneuropathy, toxic neuropathy, and physical damage to the nervous system such as that caused by physical injury of the brain and spinal cord or crush or cut injuries to the arm and hand or other parts of the body, including temporary or permanent cessation of blood flow to parts of the nervous system, as in stroke.

The NTNR α gene is expressed in muscle cells and associated neurons. Accordingly, the present invention provides for methods of treating muscle cell disorders comprising administering to a patient in need of such treatment the compounds of the invention. Muscle cell disorders which may benefit from such treatment include but are not limited to the following progressive muscular dystrophies: Duchenne, Becker, Emery-Dreifuss, Landouzy-Dejerine, scapulohumeral, limb-girdle, Von Graefe-Fuchs, oculopharyngeal, myotonic and congenital. In addition, such molecules may be of use in the treatment of congenital (central core, nemaline, centronuclear and congenital fiber-type disproportion) and acquired (toxic, inflammatory) myopathies. The present invention further provides for a method of treating a muscle cell disorder comprising administering to the patient an effective amount of NTNR α protein or an active portion thereof.

In a further embodiment of the invention, patients that suffer from an excess of NTNR, hypersensitivity to NTN, excess NTN, etc. may be treated by administering an effective amount of anti-sense RNA or anti-sense oligodeoxyribonucleotides corresponding to the NTNR gene coding region thereby decreasing expression of NTNR.

The compounds and methods of the invention may have use in conditions associated with a decrease in hematopoietic cells. Examples of these diseases include: anemia (including macrocytic and aplastic anemia); thrombocytopenia; hypoplasia; disseminated intravascular coagulation (DIC); myelodysplasia; immune (autoimmune) thrombocytopenic purpura (ITP); and HIV induced ITP. Additionally, these NTNR α molecules may be useful in treating myeloproliferative thrombocytotic diseases as well as thrombocytosis from inflammatory conditions and in iron deficiency. NTNR α polypeptide and NTNR α gene which lead to an increase in hematopoietic cell proliferation may also be used to enhance repopulation of mature blood cell lineages in cells having undergone chemo- or radiation therapy or bone marrow transplantation therapy. Generally, the NTNR α molecules are expected to lead to an enhancement of the proliferation and/or differentiation (but especially proliferation) of hematopoietic cells. Preferred embodiments provide for treatment to enhance hematopoiesis occurring in the spleen.

Other potential therapeutic applications for NTNR α and NTNR α gene include treatment to promote kidney or liver cell growth, survival, and repair. For example, acute renal failure refers to the abrupt disruption of previously normal kidney function. This serious clinical condition is due to a wide variety of mechanisms including circulatory failure (shock), vascular blockage, glomerulonephritis, and

obstruction to urine flow. Acute renal failure frequently arises as a complication of abdominal or vascular surgery. Also, low birth weight, high-risk neonates may now survive lung and heart problems due to continued improvements in prenatal care, only to die from complications of acute renal failure caused by infection or drug toxicity. Of particular clinical importance are cases of acute renal failure associated with trauma, sepsis, postoperative complications, or medication, particularly antibiotics. In particular, the compounds of the invention find use in etiologies, directly or indirectly, related to dysfunction of the enteric nervous system or renal system. Specific conditions affecting the GI include but are not limited to Achalasia, Esophageal spasm, Scleroderma (related to muscular atrophy of the smooth muscle portion of the esophagus, weakness of contraction of the lower two-thirds of the esophageal body, and incompetence of the lower esophageal sphincter, but also caused by treatment with immunosuppressive agents), disorders such as duodenal ulcer, Zollinger-Ellison Syndrome (hypersecretion of acid caused by factors including genetic factors, smoking, neural influences), hypersecretion of gastric acid, malabsorptive disorder for example, in diabetes (and hypoparathyroidism, hyperthyroidism, and adrenal insufficiency) where gastric atony, nausea, vomiting, etc. are at least in part related to dysfunction of the sympathetic/parasympathetic nervous system. Additional disorders include disorders of intestinal motility, including: Diverticulosis/diverticulitis; Hirschsprung's disease (a congenital disorder caused by absence of ganglion cells (Meissner's and Auerbach's plexuses) in a small segment of the distal colon, usually near the anus, typically presented in infants, but in less severe cases, may not be diagnosed until adolescence or early adulthood; Megacolon of other types (Hirschsprung's is a type of megacolon); Intestinal pseudo-obstruction, acute or chronic, which is a severe dysmotility due to abnormalities of sympathetic innervation of the muscle layers of the intestine, or secondarily may result from scleroderma, diabetes, amyloidosis, other neurologic diseases, drugs, or sepsis; chronic constipation, which is a serious problem in patients with mental retardation or neurological diseases, wherein a contributing factor is disordered gut motility. Also include are treatments for kidney diseases and disorders. Additional conditions include but not limited to: Spinal cord dysfunction, due to an obvious disruption of enteric nervous system; Guillain Barre syndrome; Multiple sclerosis; PANDAS (dysfunction of autonomic nervous system); Parkinsonism (frequently associated with disordered gastrointestinal motility); Multiple System Atrophy (Shy Drager Syndrome), which has been documented to have as a feature disordered gut motility; and porphyria and amyloidosis which are diffuse diseases manifested by neuropathy and often with accompanying GI motility disorders.

The necrosis or damage of NTNR-expressing or NTN-responsive tissue includes a necrosis due to microbiologic infection such as viral hepatitis, tuberculosis, typhoid fever, tularemia, brucellosis, yellow fever, and the like, or necrosis due to ischemic injury resulting from shock, heart failure, and the like, or necrosis due to acute or chronic reaction with drugs and toxic substances such as chloroform, carbon tetrachloride, phosphorous poisoning, and the like. As taught herein cellular growth enhancement, including renal cells such as renal epithelial cells and neuron innervating the kidney, is useful in treating kidney disease. The compounds and methods of the present invention provide for the repair of kidney damage. Not to be bound by theory, it is believed that this can be accomplished, either directly or indirectly, by

stimulating kidney cells, including innervating neurons, to grow and divide. Accordingly, a method for regenerating kidney tissue is provided that includes the steps of preparing a NTNR agonist (e.g. soluble NTNR α optionally complexed with NTN) as disclosed herein, optionally in combination with a pharmacologically acceptable carrier or additional growth factor or cytokine, and contacting the kidney tissue with the composition. A therapeutic amount of the composition is administered. Localized injections or implants are a preferred delivery method. Alternatively, damaged kidneys could be removed, treated ex vivo, and returned to the host after the kidney is repaired.

NTNR agonists, including NTN, can be administered during hemodialysis. Hemodialysis is defined as the temporary removal of blood from a patient for the purpose of extracting or separating toxins therefrom and the return of the cleansed blood to the same patient. Hemodialysis is indicated in patients where renal impairment or failure exists, that is, in cases where the blood is not being properly or sufficiently cleansed, (particularly to remove water) by the kidneys. In the case of chronic renal impairment or failure, hemodialysis has to be carried out on a repetitive basis. For example, in end stage kidney disease where transplantation of kidneys is not possible or for medical reasons is contra-indicated, the patient will have to be dialyzed about 100 to 150 times per year. This can result in several thousand accesses to the blood stream to enable the active hemodialysis to be performed over the remaining life of the patient.

The invention finds use in some immunosuppressive therapies where there is the side-effect of kidney damage. For example, therapy of IDDM in humans by methods designed to suppress the autoimmune response. Therapy utilizing cyclosporin A in diabetes can result in kidney damage. The invention finds use in disorders or conditions that can result in kidney damage. For example, diabetes can result in the typical late damages of blood vessels of the kidneys. Other examples include immunologically- or non-immunologically-caused kidney diseases, such as e.g. glomerulonephritis, acute kidney failure, transplant rejection and kidney damage caused by nephrotic substances, kidney transplants, toxic damage to the kidneys. Furthermore, the present invention finds use in organ transplantation, including organ transport for storing any organ enucleated from a donor to insure the protection of the organ at the time of its transplantation, minimizing any trouble occurring until the transplantation operation, and to ensure the preservation of said organ in a good condition. A preferred organ is one having NTNR-bearing or NTN-responsive cells. In one specific embodiment the organ is the kidney. Use or intervention with NTNR agonist, including NTN, promises success with regard to the maintenance of the kidney function.

As discussed herein, an object of the invention to provide methods for treatment of mammals with dysfunctional gastrointestinal muscle or disorders of smooth muscles elsewhere in the body. The gastrointestinal muscle is organized and regulated very differently than muscle elsewhere. Both skeletal and smooth muscle in the gastrointestinal tract are under the control of the enteric nervous system which is an extremely complex network of nerves and muscles, that resides within the gastrointestinal wall and orchestrates the entire digestive process including motility, secretion and absorption. The enteric nerves are also organized into interconnected networks called plexuses. Of these, the myenteric plexus, situated between the circular and longitudinal muscle layers, is the main modulator of gastrointestinal motility. It receives input from both the central nervous

system (via vagal and sympathetic pathways) as well as from local reflex pathways. Its output consists of both inhibitory and excitatory signals to the adjacent muscle. The final neural pathway regulating muscle activity in the gastrointestinal tract is therefore represented by the neurons of the myenteric plexus. A useful, if somewhat simplistic concept is to visualize net muscle tone in the gastrointestinal tract as that resulting from the balance between the opposing effects of two neuronal systems in the myenteric plexus: one causing the muscle to contract (mainly via acetylcholine) and the other causing it to relax. Both types of neurons, however, are activated by acetylcholine within the myenteric plexus. The role of acetylcholine in the regulation of gastrointestinal muscle tone is therefore complex. Acetylcholine directly released by effector nerves near the muscle causes contraction; however, within the plexus, it may result in inhibition or excitation. This is in contrast to skeletal muscle outside the gastrointestinal tract which is directly innervated by nerves emanating from the central nervous system. The interaction between nerve and muscle in skeletal muscle outside the gastrointestinal tract is far more simple: nerves release acetylcholine which causes the muscle to contract. Finally, the myenteric plexus is probably the most important but not the only determinant of muscle tone in the gastrointestinal tract. In fact, basal smooth muscle tone may be visualized as resulting from the sum of many different factors including intrinsic (myogenic) tone, and circulating hormones, in addition to nerve activity. It should be clear therefore, that the regulation of gastrointestinal tract muscle motility is far more complex than that of skeletal muscle outside the gastrointestinal tract. While there have been isolated reports on the effects of botulinum toxin on in vitro preparations of gastrointestinal smooth muscle, the regulation of gastrointestinal muscle is so complex that the physiological consequences of blocking neurotransmitter release (by using toxin such as botulinum) in humans or in live animals were not predictable prior to the present invention. The present invention provides compositions, methods, and devices for treatment of gastrointestinal disorders including achalasia, other disorders of the lower esophageal sphincter, sphincter of Oddi dysfunction, irritable bowel syndrome, and others disorders as discussed herein.

For example, provided is a method to treat Irritable Bowel Syndrome (IBS), which is a motor disorder consisting of altered bowel habits, abdominal pain, and the absence of detectable pathology. IBS is recognized by its symptoms, which are markedly influenced by psychological factors and stressful life situations. IBS is one of the most commonly encountered gastrointestinal disorders. Between 20% and 50% of patients referred to gastrointestinal clinics suffer from IBS. Symptoms of IBS occur in approximately 14% of otherwise apparently healthy people. It is a syndrome composed of a number of conditions with similar manifestations. The major symptoms of IBS (altered bowel habits, abdominal pain and bloating) are manifestations of increased motility in the gut and hyper-secretion of gastric acid. Activity of the GI tract is modulated neurally by the central nervous system (CNS) via parasympathetic and sympathetic innervation and by the peripherally located enteric nervous system (ENS) which resides within the GI tract itself.

In another aspect is provided the administration of NTNR α to a mammal having depressed levels of endogenous NTNR α or a defective NTNR α gene, preferably in the situation where such depressed levels lead to a pathological disorder, or where there is lack of activation of the NTNR α and Ret. In these embodiments where the full

length NTNR α is to be administered to the patient, it is contemplated that the gene encoding the receptor may be administered to the patient via gene therapy technology.

In gene therapy applications, genes are introduced into cells in order to achieve in vivo synthesis of a therapeutically effective genetic product, for example for replacement of a defective gene. "Gene therapy" includes both conventional gene therapy where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes in vivo. It has already been shown that short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane. (Zamecnik et al., *Proc. Natl. Acad. Sci. USA*, 83:4143-4146 (1986)). The oligonucleotides can be modified to enhance their uptake, e.g., by substituting their negatively charged phosphodiester groups by uncharged groups.

There are a variety of techniques available for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells in vitro, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred in vivo gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection (Dzau et al., *Trends in Biotechnology*, 11:205-210 (1993)). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein or the target cell, a ligand for a receptor on the target cell, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, e.g. capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu et al., *J. Biol. Chem.*, 262:4429-4432 (1987); and Wagner et al., *Proc. Natl. Acad. Sci. USA*, 87:3410-3414 (1990). For review of the currently known gene marking and gene therapy protocols see Anderson et al., *Science*, 256:808-813 (1992).

The invention also provides antagonists of NTNR α activation (e.g., NTNR α antisense nucleic acid, neutralizing antibodies). Administration of NTNR α antagonist to a mammal having increased or excessive levels of endogenous NTNR α activation is contemplated, preferably in the situation where such increased levels of NTNR α or Ret activation lead to a pathological disorder.

In one embodiment, NTNR α antagonist molecules may be used to bind endogenous ligand in the body, thereby causing desensitized NTNR α to become responsive to NTN ligand, especially when the levels of NTN ligand in the serum exceed normal physiological levels. Also, it may be beneficial to bind endogenous NTN ligand which is activating undesired cellular responses (such as proliferation of tumor cells).

Pharmaceutical compositions of the soluble NTNR α can further include a NTN or other NTNR α agonist. Such dual

compositions may be beneficial where it is therapeutically useful to prolong half-life of NTN, provide a slow-release reservoir for NTN, activate endogenous NTN α or Ret, and/or to supplement the lack of NTN α in a target Ret-expressing cell, thereby rendering the cell responsive to NTN.

Therapeutic formulations of NTN α are prepared for storage by mixing NTN α having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilizers (*Remington's Pharmaceutical Sciences*, 16th edition, Osol, A., Ed., (1980)), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as Tween, Pluronic or polyethylene glycol (PEG).

The NTN α also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively), in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules), or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences*, supra.

NTN α to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. NTN α ordinarily will be stored in lyophilized form or in solution.

Therapeutic NTN α compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of NTN α administration is in accord with known methods, e.g., those routes set forth above for specific indications, as well as the general routes of injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intercessional means, or sustained release systems as noted below. NTN α is administered continuously by infusion or by bolus injection. Generally, where the disorder permits, one should formulate and dose the NTN α for site-specific delivery. Administration can be continuous or periodic. Administration can be accomplished by a constant- or programmable-flow implantable pump or by periodic injections.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Hanger et al., *J. Biomed. Mater. Res.*, 15:167-277 (1981) and Langer, *Chem. Tech.*, 12:98-105 (1982) or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymer of L-glutamic acid and γ ethyl-L-

glutamate (Sidman et al., *Biopolymers*, 22:547-556 (1983)), non-degradable ethylene-vinyl acetate (Langer et al., supra), degradable lactic acid-glycolic acid copolymers such as the Lupron DepotTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D(-)-3-hydroxybutyric acid (EP 133,988).

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated proteins remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for protein stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release NTN α compositions also include liposomally entrapped NTN α . Liposomes containing NTN α are prepared by methods known per se: DE 3,218,121; Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688-3692 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030-4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal NTN α therapy.

When applied topically, the NTN α is suitably combined with other ingredients, such as carriers and/or adjuvants. There are no limitations on the nature of such other ingredients, except that they must be physiologically acceptable and efficacious for their intended administration, and cannot degrade the activity of the active ingredients of the composition. Examples of suitable vehicles include ointments, creams, gels, or suspensions, with or without purified collagen. The compositions also may be impregnated into transdermal patches, plasters, and bandages, preferably in liquid or semi-liquid form.

For obtaining a gel formulation, the NTN α formulated in a liquid composition may be mixed with an effective amount of a water-soluble polysaccharide or synthetic polymer such as PEG to form a gel of the proper viscosity to be applied topically. The polysaccharide that may be used includes, for example, cellulose derivatives such as etherified cellulose derivatives, including alkyl celluloses, hydroxyalkyl celluloses, and alkylhydroxyalkyl celluloses, for example, methylcellulose, hydroxyethyl cellulose, carboxymethyl cellulose, hydroxypropyl methylcellulose, and hydroxypropyl cellulose; starch and fractionated starch; agar; alginic acid and alginates; gum arabic; pullulan; agarose; carrageenan; dextrans; dextrans; fructans; inulin; mannans; xylans; arabinans; chitosans; glycogens; glucans; and synthetic biopolymers; as well as gums such as xanthan gum; guar gum; locust bean gum; gum arabic; tragacanth gum; and karaya gum; and derivatives and mixtures thereof. The preferred gelling agent herein is one that is inert to biological systems, nontoxic, simple to prepare, and not too runny or viscous, and will not destabilize the NTN α held within it.

Preferably the polysaccharide is an etherified cellulose derivative, more preferably one that is well defined, purified,

and listed in USP, e.g., methylcellulose and the hydroxyalkyl cellulose derivatives, such as hydroxypropyl cellulose, hydroxyethyl cellulose, and hydroxypropyl methylcellulose. Most preferred herein is methylcellulose.

The polyethylene glycol useful for gelling is typically a mixture of low and high molecular weight PEGs to obtain the proper viscosity. For example, a mixture of a PEG of molecular weight 400–600 with one of molecular weight 1500 would be effective for this purpose when mixed in the proper ratio to obtain a paste.

The term “water soluble” as applied to the polysaccharides and PEGs is meant to include colloidal solutions and dispersions. In general, the solubility of the cellulose derivatives is determined by the degree of substitution of ether groups, and the stabilizing derivatives useful herein should have a sufficient quantity of such ether groups per anhydroglucose unit in the cellulose chain to render the derivatives water soluble. A degree of ether substitution of at least 0.35 ether groups per anhydroglucose unit is generally sufficient. Additionally, the cellulose derivatives may be in the form of alkali metal salts, for example, the Li, Na, K, or Cs salts.

If methylcellulose is employed in the gel, preferably it comprises about 2–5%, more preferably about 3%, of the gel and the NTNR α is present in an amount of about 300–1000 mg per ml of gel.

Semipermeable, implantable membrane devices are useful as means for delivering drugs in certain circumstances. For example, cells that secrete soluble NTNR or chimeras can be encapsulated, and such devices can be implanted into a patient. For example, into the brain of patients suffering from Parkinson’s Disease. See, U.S. Pat. No. 4,892,538 of Aebischer et al.; U.S. Pat. No. 5,011,472 of Aebischer et al.; U.S. Pat. No. 5,106,627 of Aebischer et al.; PCT Application WO 91/10425; PCT Application WO 91/10470; Winn et al., *Exper. Neurology*, 113:322–329 (1991); Aebischer et al., *Exper. Neurology*, 111:269–275 (1991); and Tresco et al., *ASAIO*, 38:17–23 (1992). Accordingly, also included is a method for preventing or treating damage to a nerve or damage to other NTNR-expressing or NTN-responsive cells, e.g. kidney, as taught herein, which comprises implanting cells that secrete NTNR α , its agonists or antagonists as may be required for the particular condition, into the body of patients in need thereof. Finally, the present invention includes a device for preventing or treating nerve damage or damage to other cells as taught herein by implantation into a patient comprising a semipermeable membrane, and a cell that secretes NTNR (or its agonists or antagonists as may be required for the particular condition) encapsulated within said membrane and said membrane being permeable to NTNR (or its agonists or antagonists) and impermeable to factors from the patient detrimental to the cells. The patient’s own cells, transformed to produce NTN or NTNR ex vivo, could be implanted directly into the patient, optionally without such encapsulation. The methodology for the membrane encapsulation of living cells is familiar to those of ordinary skill in the art, and the preparation of the encapsulated cells and their implantation in patients may be accomplished without under experimentation.

The present invention includes, therefore, a method for preventing or treating nerve damage by implanting cells, into the body of a patient in need thereof, cells either selected for their natural ability to generate NTNR α or engineered to secrete NTNR α . Preferably, the secreted NTNR α being soluble, human mature NTNR α when the patient is human. The implants are preferably non-immunogenic and/or prevent immunogenic implanted cells

from being recognized by the immune system. For CNS delivery, a preferred location for the implant is the cerebral spinal fluid of the spinal cord.

An effective amount of NTNR α to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the optimal therapeutic effect. Typically, the clinician will administer the NTNR α until a dosage is reached that achieves the desired effect. A typical daily dosage for systemic treatment might range from about 1 μ g/kg to up to 10 mg/kg or more, depending on the factors mentioned above. As an alternative general proposition, the NTNR α is formulated and delivered to the target site or tissue at a dosage capable of establishing in the tissue a NTNR α level greater than about 0.1 ng/cc up to a maximum dose that is efficacious but not unduly toxic. This intra-tissue concentration should be maintained if possible by continuous infusion, sustained release, topical application, NTNR α -expressing cell implant, or injection at empirically determined frequencies. The progress of this therapy is easily monitored by conventional assays. When administered in complex with or concomitantly with NTN, a 100:1 to 1:100 ratio of NTNR α to NTN dimer is useful. Preferably the ratio is 10:1 to 1:10, more preferably 1:1, and even more preferably 2:1, which may reflect the natural binding ratio of NTNR α to NTN. An amount of NTN effective to produce the desired result is administered. A typical daily dosage for systemic treatment might also range from about 1 μ g/kg to up to 10 mg/kg or more, depending on the factors mentioned above.

NTNR α nucleic acid is useful for the preparation of NTNR α polypeptide by recombinant techniques exemplified herein which can then be used for production of anti-NTNR α antibodies having various utilities described below.

The NTNR α (polypeptide or nucleic acid) can be used to increase NrN-responsiveness (and thus increase cell survival and modulate Ret-mediated downstream pathways) of cells in vitro. Such cells must contain or be modified to contain cell surface Ret. Cultured ex vivo, these cells may simultaneously be exposed to other known neurotrophic factors or cytokines, such as those described herein.

In yet another aspect of the invention, the NTNR α may be used for affinity purification of ligands that bind to the NTNR α , either naturally-occurring or synthetic ligands. NTN is a preferred ligand for purification. Briefly, this technique involves: (a) contacting a source of NTN ligand with an immobilized NTNR α under conditions whereby the NTN ligand to be purified is selectively adsorbed onto the immobilized receptor; (b) washing the immobilized NTNR α and its support to remove non-adsorbed material; and (c) eluting the NTN ligand molecules from the immobilized NTNR α to which they are adsorbed with an elution buffer. In a particularly preferred embodiment of affinity purification, NTNR α is covalently attaching to an inert and porous matrix or resin (e.g., agarose reacted with cyanogen bromide). Especially preferred is a NTNR α immunoadsorbent immobilized on a protein A column. A solution containing NTN ligand is then passed through the chromatographic material. The NTN ligand adsorbs to the column and is subsequently released by changing the elution conditions (e.g. by changing pH or ionic strength). Novel ligands can be detected by monitoring displacement of a known, labelled NTNR α ligand, such as I¹²⁵- or biotinylated-NTN.

The NTNR α may be used for competitive screening of potential agonists or antagonists for binding to the NTNR α .

Such agonists or antagonists may constitute potential therapeutics for treating conditions characterized by insufficient or excessive NTNR α activation, respectively.

The preferred technique for identifying molecules which bind to the NTNR α utilizes a chimeric receptor (e.g., epitope-tagged NTNR α or NTNR α immunoadhesin) attached to a solid phase, such as the well of an assay plate. The binding of the candidate molecules, which are optionally labelled (e.g., radiolabeled), to the immobilized receptor can be measured. Alternatively, competition for binding of a known, labelled NTNR α ligand, such as I¹²⁵-NTN, can be measured. For screening for antagonists, the NTNR α can be exposed to a NTN ligand followed by the putative antagonist, or the NTN ligand and antagonist can be added to the NTNR α simultaneously, and the ability of the antagonist to block receptor activation can be evaluated.

The present invention also provides for assay systems for detecting NTN activity, comprising cells which express high levels of NTNR α , and which are, therefore, extremely sensitive to even very low concentrations of NTN or NTN-like molecules. The present invention provides for assay systems in which NTN activity or activities similar to NTN activity resulting from exposure to a peptide or non-peptide compound may be detected by measuring a physiological response to NTN in a cell or cell line responsive to NTN which expresses the NTNR molecules of the invention. A physiological response may comprise any of the biological effects of NTN, including but not limited to, those described herein, as well as the transcriptional activation of certain nucleic acid sequences (e.g. promoter/enhancer elements as well as structural genes), NTN-related processing, translation, or phosphorylation, the induction of secondary processes in response to processes directly or indirectly induced by NTN, including Ret-mediated effects, and morphological changes, such as neurite sprouting, or the ability to support the survival of cells, for example, nodose or dorsal root ganglion cells, motoneurons, dopaminergic neurons, sensory neurons. Purkinje cells, or hippocampal neurons.

In one embodiment of the invention, the functional interaction between NTN and the NTNR α may be observed by detecting an increase in the production of autophosphorylated Ret protein, or alternatively, phosphorylated ERK-1 or ERK-2 homologs (See Kotzbauer et al., supra).

The present invention provides for the development of novel assay systems which can be utilized in the screening of compounds for NTN- or NTN-like activity. Target cells which bind NTN may be produced by transfection with NTNR α -encoding nucleic acid or may be identified and segregated by, for example, fluorescent-activated cell sorting, sedimentation of rosettes, or limiting dilution. Once target cell lines are produced or identified, it may be desirable to select for cells which are exceptionally sensitive to NTN. Such target cells may bear a greater number of NTNR α molecules; target cells bearing a relative abundance of NTNR α can be identified by selecting target cells which bind to high levels of NTN, for example, by marking high-expressors with fluorophore tagged-NTN followed by immunofluorescence detection and cell sorting. Alternatively, cells which are exceptionally sensitive to NTN may exhibit a relatively strong biological response in response to NTN binding, such as a sharp increase in Ret-mediated effects or in immediate early gene products such as c-fos or c-jun. By developing assay systems using target cells which are extremely sensitive to NTN, the present invention provides for methods of screening for NTN or NTN-like activity which are capable of detecting low levels of NTN activity.

In particular, using recombinant DNA techniques, the present invention provides for NTN target cells which are engineered to be highly sensitive to NTN. For example, the NTN-receptor gene can be inserted into cells which are naturally NTN responsive such that the recombinant NTNR gene is expressed at high levels and the resulting engineered target cells express a high number of NTNRs on their cell surface. Alternatively, or additionally, the target cells may be engineered to comprise a recombinant gene which is expressed at high levels in response to NTN/receptor binding. Such a recombinant gene may preferably be associated with a readily detectable product. For example, and not by way of limitation, transcriptional control regions (i.e. promoter/enhancer regions) from an immediate early gene may be used to control the expression of a reporter gene in a construct which may be introduced into target cells. The immediate early gene/reporter gene construct, when expressed at high levels in target cells by virtue of a strong promoter/enhancer or high copy number, may be used to produce an amplified response to NTNR binding. For example, and not by way of limitation, a NTN-responsive promoter may be used to control the expression of detectable reporter genes including β -galactosidase, growth hormone, chloramphenicol acetyl transferase, neomycin phosphotransferase, luciferase, or β -glucuronidase. Detection of the products of these reporter genes, well known to one skilled in the art, may serve as a sensitive indicator for NTN or NTN-like activity of pharmaceutical compounds.

The NTNR α -encoding or reporter gene constructs discussed herein (e.g., soluble ECD) can be inserted into target cells using any method known in the art, including but not limited to transfection, electroporation, calcium phosphate/DEAE dextran methods, and cell gun. The constructs and engineered target cells can be used for the production of transgenic animals bearing the above-mentioned constructs as transgenes, from which NTNR α -expressing target cells may be selected using the methods discussed.

Nucleic acids which encode NTNR, preferably from non-human species, such as murine or rat protein, can be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic, stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, the human and/or rat cDNA encoding NTNR α , or an appropriate sequence thereof, can be used to clone genomic DNA encoding NTNR in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding NTNR. Methods for generating transgenic animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Pat. Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for NTNR transgene incorporation with tissue-specific enhancers, which could result in desired effect of treatment. Transgenic animals that include a copy of a transgene encoding NTNR introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding NTNR. Such animals can be used as tester animals for reagents thought to confer protection from, for example, diseases related to NTN. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the disease, compared to

untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the disease.

Transgenic mice bearing minigenes are currently preferred. First a fusion enzyme expression construct is created and selected based on expression in cell culture as described in the Examples. Then a minigene capable of expressing that fusion enzyme is constructed using known techniques. Particularly preferred hosts are those bearing minigene constructs comprising a transcriptional regulatory element that is tissue-specific for expression.

Transgenic mice expressing NTNR minigene are made using known techniques, involving, for example, retrieval of fertilized ova, microinjection of the DNA construct into male pronuclei, and re-insertion of the fertilized transgenic ova into the uteri of hormonally manipulated pseudopregnant foster mothers. Alternatively, chimeras are made using known techniques employing, for example, embryonic stem cells (Rossant et al., *Philos. Trans. R. Soc. Lond. Biol.* 339:207–215 (1993)) or primordial germ cells (Vick et al. *Philos. Trans. R. Soc. Lond. Biol.* 251:179–182 (1993)) of the host species. Insertion of the transgene can be evaluated by Southern blotting of DNA prepared from the tails of offspring mice. Such transgenic mice are then back-crossed to yield homozygotes.

It is now well-established that transgenes are expressed more efficiently if they contain introns at the 5' end, and if these are the naturally occurring introns (Brinster et al. *Proc. Natl. Acad. Sci. USA* 85:836 (1988); Yokode et al., *Science* 250:1273 (1990)).

Transgenic mice expressing NTNR minigene are created using established procedures for creating transgenic mice. Transgenic mice are constructed using now standard methods (et al. *Proc. Natl. Acad. Sci. USA* 85:836 (1988); Yokode et al., *Science* 250:1273 (1990); Rubin et al., *Proc Natl Acad Sci USA* 88:434 (1991); Rubin et al. *Nature* 353:265 (1991)). Fertilized eggs from timed matings are harvested from the oviduct by gentle rinsing with PBS and are microinjected with up to 100 nanoliters of a DNA solution, delivering about 10^4 DNA molecules into the male pronucleus. Successfully injected eggs are then re-implanted into pseudopregnant foster mothers by oviduct transfer. Less than 5% of microinjected eggs yield transgenic offspring and only about $\frac{1}{3}$ of these actively express the transgene: this number is presumably influenced by the site at which the transgene enters the genome.

Transgenic offspring are identified by demonstrating incorporation of the microinjected transgene into their genomes, preferably by preparing DNA from short sections of tail and analyzing by Southern blotting for presence of the transgene ("Tail Blots"). A preferred probe is a segment of a minigene fusion construct that is uniquely present in the transgene and not in the mouse genome. Alternatively, substitution of a natural sequence of codons in the transgene with a different sequence that still encodes the same peptide yields a unique region identifiable in DNA and RNA analysis. Transgenic "founder" mice identified in this fashion are bred with normal mice to yield heterozygotes, which are back-crossed to create a line of transgenic mice. Tail blots of each mouse from each generation are examined until the strain is established and homozygous. Each successfully created founder mouse and its strain vary from other strains in the location and copy number of transgenes inserted into the mouse genome, and hence have widely varying levels of transgene expression. Selected animals from each established line are sacrificed at 2 months of age and the expression of the transgene is analyzed by Northern blotting of

RNA from liver, muscle, fat, kidney, brain, lung, heart, spleen, gonad, adrenal and intestine.

Alternatively, the non-human homologs of NTNR can be used to construct a NTNR "knock out" animal, i.e., having a defective or altered gene encoding NTNR, as a result of homologous recombination between the endogenous NTNR gene and an altered genomic NTNR DNA introduced into an embryonic cell of the animal. For example, murine NTNR cDNA can be used to clone genomic NTNR DNA in accordance with established techniques. A portion of the genomic NTNR DNA (e.g., such as an exon which encodes e.g., an extracellular domain) can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas and Capecchi, *Cell* 51:503 (1987) for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see e.g., Li et al., *Cell* 69:915 (1992)). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113–152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for their ability to accept grafts, reject tumors and defend against infectious diseases and can be used in the study of basic immunobiology.

In addition to the above procedures, which can be used for preparing recombinant DNA molecules and transformed host animals in accordance with the practices of this invention, other known techniques and modifications thereof can be used in carrying out the practice of the invention. For example, U.S. Pat. No. 4,736,866 discloses vectors and methods for production of a transgenic non-human eukaryotic animal whose germ cells and somatic cells contain a gene sequence introduced into the animal, or an ancestor of the animal, at an embryonic stage. U.S. Pat. No. 5,087,571 discloses a method of providing a cell culture comprising (1) providing a transgenic non-human mammal, all of whose germ cells and somatic cells contain a recombinant gene sequence introduced at an embryonic stage; and (2) culturing one or more of said somatic cells. U.S. Pat. No. 5,175,385 discloses vectors and methods for production of a transgenic mouse whose somatic and germ cells contain and express a gene at sufficient levels to provide the desired phenotype in the mouse, the gene having been introduced into said mouse or an ancestor of said mouse at an embryonic stage, preferably by microinjection. A partially constitutive promoter, the metallothionein promoter, was used to drive heterologous gene expression. U.S. Pat. No. 5,175,384 discloses a method of introducing a transgene into an embryo by infecting the embryo with a retrovirus containing the transgene. U.S. Pat. No. 5,175,383 discloses DNA constructs having a gene, homologous to the host cell, operably linked to a heterologous and inducible promoter effective for the expression of the gene in the urogenital tissues of a mouse, the transgene being introduced into the mouse at an embryonic stage to produce a transgenic mouse.

Even though a homologous gene is introduced, the gene can integrate into a chromosome of the mouse at a site different from the location of the endogenous coding sequence. The vital MMTV promoter was disclosed as a suitable inducible promoter. U.S. Pat. No. 5,162,215 discloses methods and vectors for transfer of genes in avian species, including livestock species such as chickens, turkeys, quails or ducks, utilizing pluripotent stem cells of embryos to produce transgenic animals. U.S. Pat. No. 5,082,779 discloses pituitary-specific expression promoters for use in producing transgenic animals capable of tissue-specific expression of a gene. U.S. Pat. No. 5,075,229 discloses vectors and methods to produce transgenic, chimeric animals whose hemopoietic liver cells contain and express a functional gene driven by a liver-specific promoter, by injecting into the peritoneal cavity of a host fetus the disclosed vectors such that the vector integrates into the genome of fetal hemopoietic liver cells.

Although some of the above-mentioned patents and publications are directed to the production or use of a particular gene product or material that are not within the scope of the present invention, the procedures described therein can easily be modified to the practice of the invention described in this specification by those skilled in the art of fermentation and genetic engineering.

Assay systems of the present invention enable the efficient screening of pharmaceutical compounds for use in the treatment of NTN-associated diseases. For example, and not by way of limitation, it may be desirable to screen a pharmaceutical agent for NTN activity and therapeutic efficacy in cerebellar degeneration. In a one embodiment of the invention, cells responsive to NTN may be identified and isolated, and then cultured in microwells in a multiwell culture plate. Culture medium with added test agent, or added NTN, in numerous dilutions may be added to the wells, together with suitable controls. The cells may then be examined for improved survival, neurite sprouting, and the like, and the activity of test agent and NTN, as well as their relative activities, can be determined. For example, one can now identify NTN-like compounds which can, like NTN, prevent motoneuron cell death in response to toxic assault or axotomy, for example. NTN responsive motoneurons could be utilized in assay systems to identify compounds useful in treating motoneuron diseases. If a particular disease is found to be associated with a defective NTN response in a particular tissue, a rational treatment for the disease would be supplying the patient with exogenous NTN. However, it may be desirable to develop molecules which have a longer half-life than endogenous NTN, or which act as NTN agonists, or which are targeted to a particular tissue. Accordingly, the methods of the invention can be used to produce efficient and sensitive screening systems which can be used to identify molecules with the desired properties. Similar assay systems could be used to identify NTN antagonists.

In addition, the present invention provides for experimental model systems for studying the physiological role of NTN and its receptor. Such systems include animal models, such as (i) animals exposed to circulating NTN α peptides which compete with cellular receptor for NTN binding and thereby produce a NTN-depleted condition, (ii) animals immunized with NTN α ; (iii) transgenic animals which express high levels of NTN α and therefore are hypersensitive to NTN; and (iv) animals derived using embryonic stem cell technology in which the endogenous NTN α genes were deleted from the genome.

The present invention also provides for experimental model systems for studying the physiological role of NTN

and its receptor. In these model systems NTN α protein, peptide fragment, or a derivative thereof, may be either supplied to the system or produced within the system. Such model systems could be used to study the effects of NTN excess or NTN depletion. The experimental model systems may be used to study the effects of increased or decreased response to NTN in cell or tissue cultures, in whole animals, in particular cells or tissues within whole animals or tissue culture systems, or over specified time intervals (including during embryogenesis) in embodiments in which NTN α expression is controlled by an inducible or developmentally regulated promoter. In a particular embodiment of the invention, the CMV promoter may be used to control expression of NTN α in transgenic animals. Transgenic animals, as discussed herein, are produced by any method known in the art, including, but not limited to microinjection, cell fusion, transfection, and electroporation.

The present invention also provides for model systems for autoimmune disease in which an autoimmune response is directed toward NTN α . Such models comprise animals which have been immunized with immunogenic amounts of NTN α and preferably found to produce anti-NTN α antibodies and/or cell-mediated immunity. To produce such a model system, it may be desirable to administer the NTN α in conjunction with an immune adjuvant.

For example, and not by way of limitation, an experimental model system may be created which may be used to study the effects of excess NTN activity. In such a system, the response to NTN may be increased by engineering an increased number of NTN α s on cells of the model system relative to cells which have not been so engineered. These cells should also express Ret or another signalling molecule capable of interacting with NTN α and mediating an NTN signal. It may be preferable to provide an increased number of NTN α s selectively on cells which normally express NTN α s. Cells may be engineered to produce increased numbers of NTN α by infection with a virus which carries a NTN α gene of the invention. Alternatively, the NTN α gene may be provided to the cells by transfection. If the model system is an animal, a recombinant NTN α gene may be introduced into the cells of the animal by infection with a virus which carries the NTN α gene or other means as discussed herein. For example, a transgenic animal may be created which carries the NTN α gene as a transgene. In order to ensure expression of NTN α , the NTN α gene should be placed under the control of a suitable promoter sequence. It may be desirable to put the NTN α gene under the control of a constitutive and/or tissue specific promoter. By increasing the number of cellular NTN α s, the response to endogenous NTN may be increased. If the model system contains little or no NTN, NTN may be added to the system. It may also be desirable to add additional NTN to the model system in order to evaluate the effects of excess NTN activity. Over expressing NTN (or secreted NTN) may be the preferable method for studying the effects of elevated levels of NTN on cells already expressing NTN α . More preferably would be to express NTN α in all cells (general expression) and determine which cells are then endowed with functional responsiveness to NTN, thus allowing the potential identification of a second receptor component, if one exists.

An experimental model system may be created which may be used to study the effects of diminished NTN activity. This system may permit identification of processes or neurons which require NTN, and which may represent potential therapeutic targets. In such a system, the response to NTN may be decreased by providing recombinant NTN α s which are not associated with a cell surface or which are engi-

neered so as to be ineffective in transducing a response to NTN. For example, NTNR protein, peptide, or derivative may be supplied to the system such that the supplied receptor may compete with endogenous NTNR for NTN binding, thereby diminishing the response to NTN. The NTNR may be a cell free receptor which is either added to the system or produced by the system. For example, a NTNR protein which lacks the transmembrane domain may be produced by cells within the system, such as an anchorless NTNR that may be secreted from the producing cell. Alternatively, NTNR protein, peptide or derivative may be added to an extracellular space within the system. In additional embodiments of the invention, a recombinant NTNR gene may be used to inactivate or "knock out" the endogenous gene by homologous recombination, and thus create a NTNR deficient cell, tissue, or animal. For example, and not by way of limitation, a recombinant NTNR gene may be engineered to contain an insertional mutation, for example the neo gene, which inactivates NTNR. Such a construct, under the control of a suitable promoter, may be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, transduction, injection, etc. Cells containing the construct may then be selected by G418 resistance. Cells which lack an intact NTNR gene may then be identified, e.g. by Southern blotting or Northern blotting or assay of expression. Cells lacking an intact NTNR gene may then be fused to early embryo cells to generate transgenic animals deficient in NTNR. A comparison of such an animal with an animal not expressing endogenous NTN would reveal that either the two phenotypes match completely or that they do not, implying the presence of additional NTN-like factors or receptors. Such an animal may be used to define specific cell populations, e.g., neuronal populations, or any other *in vivo* processes, normally dependent upon NTN or its receptor. Thus, these populations or processes may be expected to be effected if the animal did not express NTNR and therefore could not respond to NTN. Alternatively, a recombinant NTNR protein, peptide, or derivative which competes with endogenous receptor for NTN may be expressed on the surface of cells within the system, but may be engineered so as to fail to transduce a response to NTN binding. The recombinant NTNR proteins, peptides or derivatives described above may bind to NTN with an affinity that is similar to or different from the affinity of endogenous NTNR to NTN. To more effectively diminish the response to NTN, the NTNR protein, peptide, or derivative may desirably bind to NTN with a greater affinity than that exhibited by the native receptor. If the NTNR protein, peptide, or derivative is produced within the model system, nucleic acid encoding the NTNR protein, peptide, or derivative may be supplied to the system by infection, transduction, transfection, etc. or as a transgene. As discussed supra, the NTNR gene may be placed under the control of a suitable promoter, which may be, for example, a tissue-specific promoter or an inducible promoter or developmentally regulated promoter. In a specific embodiment of the invention the endogenous NTNR gene of a cell may be replaced by a mutant NTNR gene by homologous recombination. In a further embodiment of the invention, NTNR expression may be reduced by providing NTNR expressing cells with an amount of NTNR antisense RNA or DNA effective to reduce expression of NTNR protein.

The polypeptides of the invention also find use as feed additives for animals. The nucleic acids of the invention find use in preparing these polypeptides.

The NTNR α polypeptides are also useful as molecular weight markers. To use a NTNR α polypeptide as a molecu-

lar weight marker, gel filtration chromatography or SDS-PAGE, for example, will be used to separate protein(s) for which it is desired to determine their molecular weight(s) in substantially the normal way. NTNR α , preferably a soluble NTNR, and other molecular weight markers will be used as standards to provide a range of molecular weights. For example, phosphorylase b (mw=97,400), bovine serum albumin (mw=68,000), ovalbumin (mw=46,000), trypsin inhibitor (mw=20,100), and lysozyme (mw=14,400) can be used as MW markers. The other molecular weight markers mentioned here can be purchased commercially from Amersham Corporation, Arlington Heights, Ill. The molecular weight markers are generally labeled to facilitate detection thereof. For example, the markers may be biotinylated and, following separation, can be incubated with streptavidin-horseradish peroxidase so that the various markers can be detected by light detection.

The purified NTNR α , and the nucleic acid encoding it, may also be sold as reagents for mechanism studies of NTNR α and its ligands, to study the role of the NTNR α and NTN ligand in normal growth and development, as well as abnormal growth and development, e.g., in malignancies. NTNR probes can be used to identify cells and tissues which are responsive to NTN in normal or diseased states. For example, a patient suffering from a NTN-related disorder may exhibit an aberrancy of NTNR expression. The present invention provides for methods for identifying cells which are responsive to NTN comprising detecting NTNR expression in such cells. NTNR expression may be evidenced by transcription of NTNR mRNA or production of NTNR protein. NTNR expression may be detected using probes which identify NTNR nucleic acid or protein. One variety of probe which may be used to detect NTNR expression is a nucleic acid probe, which may be used to detect NTNR-encoding RNA by any method known in the art, including, but not limited to, *in situ* hybridization, Northern blot analysis, or PCR related techniques. Another variety of probe which may be used is tagged NTN as discussed herein.

According to the invention, tagged NTN may be incubated with cells under conditions which would promote the binding or attachment of NTN to said cells. In most cases, this may be achieved under standard culture conditions. For example, in one embodiment of the invention, cells may be incubated for about 30 minutes in the presence of tagged NTN. If the tag is an antibody molecule, it may be preferable to allow NTN to bind to cells first and subsequently wash cells to remove unbound ligand and then add anti-NTN antibody tag. In another embodiment of the invention, tagged NTN on the surface of NTN-responsive cells, hereafter called target cells, may be detected by rosetting assays in which indicator cells that are capable of binding to the tag are incubated with cells bearing tagged-NTN such that they adhere to tagged-NTN on the target cells and the bound indicator cells form rosette-like clusters around NTN-tag bearing cells. These rosettes may be visualized by standard microscopic techniques on plated cells, or, alternatively, may allow separation of rosetted and non-rosetted cells by density centrifugation. In a preferred specific embodiment of the invention, target cells, such as neuronal cells. In alternative embodiments of the invention, tagged-NTN on the surface of target cells may be detected using immunofluorescent techniques in which a molecule which reacts with the tag, preferably an antibody, directly or indirectly produces fluorescent light. The fluorescence may either be observed under a microscope or used to segregate tagged-NTN-bearing cells by fluorescence activated cell sorting

techniques. The present invention also provides for methods for detecting other forms of tags, such as chromogenic tags and catalytic tags. An anti-NTNR antibody can also be used as a probe. The detection methods for any particular tag will depend on the conditions necessary for producing a signal from the tag, but should be readily discernible by one skilled in the art.

NTNR α variants are useful as standards or controls in assays for the NTNR α for example ELISA, RIA, or RRA, provided that they are recognized by the analytical system employed, e.g., an anti-NTNR α antibody.

Polyclonal antibodies are generally raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and all adjuvant. In that the preferred epitope is in the ECD of the NTNR α , it is desirable to use NTNR α ECD or a molecule comprising the ECD (e.g., NTNR α immunoadhesin) as the antigen for generation of polyclonal and monoclonal antibodies. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl₂, or R¹N=C=NR, where R and R¹ are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining 1 mg or 1 μ g of the peptide or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal antibodies are obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies.

For example, the monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (Cabilly et al., supra).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or

more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson et al., *Anal. Biochem.*, 107:220 (1980).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, supra). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylamine chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The ability of the MAbs to block binding of NTN to its receptor can be evaluated by ELISA and bioassay utilizing available reagents (rhNTNr-IgG; a stable transfected CHO cell line expressing NTNR α). Neutralizing activities can also be evaluated by neuronal survival assay(s).

NTNR-specific MAbs can be developed as discussed above using for example, the receptor immunoadhesin and transfected cell line) to initiate new immunization protocols to generate NTNR-specific MAbs for use as potential agonists or antagonists, as well as for immunohistochemistry, immunocytochemistry, and assay development. The MAbs generated from fusion of the immunized animals can be screened for agonist and antagonist activities by bioassay (e.g., neuron survival assays, signal transduction/phosphorylation, kidney cell survival assays) as well as by ELISA and FACS (functional blocking of NTN-NTNR binding). Suitable techniques are provided in, for example, Lucas et al, *J. Immunol.* 145:1415-1422(1990); Hoogenraad et al. *J. Immunol. Methods* 6:317-320(1983); Moks et al., *Eur. J. Biochem.* 85:1205-1210 (1986); Laemmli, *Nature*

(London) 227:680–685 (1970); and, Towbin et al., *Proc Natl Acad Sci USA* 76:4350–4354 (1979).

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Review articles on recombinant expression in bacteria of DNA encoding the antibody include Skerra et al., *Curr. Opin. Immunol.*, 5:256–262 (1993) and Pluckthun, *Immunol. Revs.*, 130:151–188 (1992).

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature*, 348:552–554 (1990). Clackson et al., *Nature*, 352:624–628 (1991) and Marks et al., *J. Mol. Biol.*, 222:581–597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Mark et al., *Bio/Technology*, 10:779–783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.*, 21:2265–2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (Cabilly et al., supra; Morrison, et al., *Proc. Nat. Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

Chimeric or hybrid antibodies also may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide-exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate.

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as “import” residues, which are typically taken from an “import” variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature*, 321:522–525 (1986); Riechmann et al., *Nature*, 332:323–327 (1988); Verhoeyen et al., *Science*, 239:15534–1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such

“humanized” antibodies are chimeric antibodies (Cabilly et al., supra), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a nonhuman species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called “best-fit” method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims et al., *J. Immunol.*, 151:2296 (1993); Chothia et al., *J. Mol. Biol.*, 196:901 (1987)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA*, 89:4285(1992); Presta et al., *J. Immunol.*, 151:2623(1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the consensus and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits et al., *Nature*, 362:255–258 (1993); Bruggermann et al., *Year in Immunol.*, 7:33 (1993). Human antibodies can also be produced in phage-display libraries (Hoogenboom et al., *J. Mol. Biol.*, 227:381 (1991); Marks et al., *J. Mol. Biol.*, 222:581 (1991)).

Bispecific antibodies (BsAbs) are antibodies that have binding specificities for at least two different antigens. BsAbs can be used as tumor targeting or imaging agents and can be used to target enzymes or toxins to a cell possessing the NTNR α . Such antibodies can be derived from full length

antibodies or antibody fragments (e.g. F(ab')₂ bispecific antibodies). In accordance with the present invention, the BsAb may possess one arm which binds the NTNR α and another arm which binds to a cytokine or another cytokine receptor (or a subunit thereof) such as the receptors for TPO, EPO, G-CSF, IL-4, IL-7, GH, PRL; the α or β subunits of the IL-3, GM-CSF, IL-5, IL-6, LIF, OSM and CNTF receptors; or the α , β or γ subunits of the IL-2 receptor complex. For example, the BsAb may bind both NTNR α and gp130.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, published May 13, 1993, and in Traunecker et al., *EMBO J.*, 10:3655-3659 (1991).

According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH₂, and CH₃ regions. It is preferred to have the first heavy-chain constant region (CH₁) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published Mar. 3, 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., *Methods in Enzymology*, 121:210 (1986).

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking

methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. The following techniques can also be used for the production of bivalent antibody fragments which are not necessarily bispecific. According to these techniques, Fab'-SH fragments can be recovered from *E. coli*, which can be chemically coupled to form bivalent antibodies. Shalaby et al., *J. Exp. Med.*, 175:217-225 (1992) describe the production of a fully humanized BsAb F(ab')₂ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the BsAb. The BsAb thus formed was able to bind to cells overexpressing the HER2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets. See also Rodrigues et al., *Int. J. Cancers*, (Suppl.) 7:45-50 (1992).

Various techniques for making and isolating bivalent antibody fragments directly from recombinant cell culture have also been described. For example, bivalent heterodimers have been produced using leucine zippers. Kostelny et al., *J. Immunol.*, 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA*, 90:6444-6448 (1993) has provided an alternative mechanism for making BsAb fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making BsAb fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.*, 152:5368 (1994).

The NTNR α agonists (including NTN) and agonist NTNR α antibodies of the present invention can be used to enhance splenic hematopoiesis, allowing some repopulation of blood cell lineages in patients having undergone chemotherapy or radiation therapy and transplantation. Generally, the antibodies will act to enhance proliferation and/or differentiation (but especially proliferation) of hematopoietic cells in the spleen. Without being bound by theory, NTNR agonists may act directly as a growth, survival or differentiation factor for hematopoietic cells in the spleen and/or may indirectly act on the splenic stromal environment (possibly neurons involved in the splenic innervation) to produce another factor that is responsible for the maintenance of hematopoietic lineages. In any event, as taught herein NTNR agonist, including NTN, have therapeutic benefit in facilitating the splenic engraftment of bone marrow transplants following irradiation or chemotherapy or for stimulating extramedullary hematopoiesis in the spleen (which is normal in rodents, but not normally seen in man) in those conditions where there is an increased demand for blood cell production due to anemia (red blood cells), chronic infection (neutrophils), bone marrow failure (all lineages), and immune deficiency (lymphocytes). The agonists may similarly be useful for treating diseases characterized by a decrease in blood cells. Examples of these diseases include: anemia (including macrocytic and aplastic anemia); throm-

bocytopenia; hypoplasia; immune (autoimmune) thrombocytopenic purpura (ITP); and HIV induced ITP. Also, the agonists may be used to treat a patient having suffered a hemorrhage.

Therapeutic applications for NTN α neutralizing antibodies include the treatment of metabolic disorders and cell tumors at sites of NTN α expression, especially those tumors characterized by overexpression of NTN α .

For therapeutic applications, the NTN α antibodies of the invention are administered to a mammal, preferably a human, in a physiologically acceptable dosage form, including those that may be administered to a human intravenously as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intra-cerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, oral, topical, or inhalation routes. The NTN α antibodies also are suitably administered by intratumoral, peritumoral, intralesional, or perilesional routes or to the lymph, to exert local as well as systemic therapeutic effects.

Such dosage forms encompass physiologically acceptable carriers that are inherently non-toxic and non-therapeutic. Examples of such carriers include ion exchangers, alumina, aluminum stearate, lecithin, serum proteins, such as human serum albumin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, partial glyceride mixtures of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, zinc salts, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-based substances, and PEG. Carriers for topical or gel-based forms of NTN α antibodies include polysaccharides such as sodium carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, polyoxyethylene-polyoxypropylene-block polymers, PEG, and wood wax alcohols. For all administrations, conventional depot forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, plasters, inhalation forms, nose sprays, sublingual tablets, and sustained-release preparations. The NTN α antibody will typically be formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml.

Suitable examples of sustained-release preparations include semipenetrable matrices of solid hydrophobic polymers containing the NTN α antibody, which matrices are in the form of shaped articles, e.g. films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate) as described by Langer et al., supra and Langer, supra, or poly(vinylalcohol), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate (Sidman et al., supra), non-degradable ethylene-vinyl acetate (Langer et al., supra), degradable lactic acid-glycolic acid copolymers such as the Luproni DepotTM (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated NTN α antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37° C., resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S—S bond formation through thio-disulfide interchange, stabilization may be achieved by modifying

sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release NTN α antibody compositions also include liposomally entrapped antibodies. Liposomes containing the NTN α antibodies are prepared by methods known in the art, such as described in Epstein et al., *Proc. Natl. Acad. Sci. USA*, 82:3688 (1985); Hwang et al., *Proc. Natl. Acad. Sci. USA*, 77:4030 (1980); and U.S. Pat. Nos. 4,485,045 and 4,544,545. Ordinarily, the liposomes are the small (about 200–800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. % cholesterol, the selected proportion being adjusted for the optimal NTN α antibody therapy. Liposomes with enhanced circulation time are disclosed in U.S. Pat. No. 5,013,556.

For the prevention or treatment of disease, the appropriate dosage of NTN α antibody will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the antibodies are administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the NTN α antibody, and the discretion of the attending physician. The NTN α antibody is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 μ g/kg to 15 mg/kg of NTN α antibody is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A typical daily dosage might range from about 1 μ g/kg to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

Animal model are available to assess effects of the compounds and method of the invention. For example, to assess the effects of treating damaged kidneys with compositions that affect growth (Toback, 1977; Toback et al. 1977), an intravenous injection of 1.0 to 1.1 mg of mercury per kg of body weight as HgCl₂ is given to rats to induce a reversible syndrome of acute nonoliguric acute renal failure. After one day, there are marked increases in serum urea nitrogen concentration (SUN), urinary excretion of sodium and protein, and necrosis of proximal tubular cells. By day two, increases in phospholipid, DNA and RNA synthesis, and mitotic index indicate that cellular regeneration is underway. By day three, the SUN reaches a maximum, and squamoid epithelial cells appear on the tubular basement membrane. At day five, the SUN returns to normal, the maximal rate of phospholipid synthesis is reached, and the tubules are repopulated with more mature cells. The effects of infusion of a composition of autocrine growth factors on renal structure is compared with untreated rats and animals infused with vehicle alone during the course of the mercuric chloride-induced acute tubular necrosis syndrome discussed above.

The NTN α antibodies of the invention are also useful as affinity purification agents. In this process, the antibodies against NTN α are immobilized on a suitable support, such as a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the NTN α to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except

the NTNR α , which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the NTNR α from the antibody.

NTNR α antibodies may also be useful in diagnostic assays for NTNR α , e.g., detecting its expression in specific cells, tissues, or serum. For diagnostic applications, antibodies typically will be labeled with a detectable moiety. The detectable moiety can be any one which is capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^4C , ^{32}P , ^{35}S , or ^{125}I ; a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin; radioactive isotopic labels, such as, e.g., ^{125}I , ^{32}P , ^{14}C , or ^3H ; or an enzyme, such as alkaline phosphatase, beta-galactosidase, or horseradish peroxidase.

Any method known in the art for separately conjugating the polypeptide variant to the detectable moiety may be employed, including those methods described by Hunter et al., *Nature*, 144:945 (1962); David et al., *Biochemistry*, 13:1014 (1974); Pain et al., *J. Immunol. Meth.*, 40:219 (1981); and Nygren, *J. Histochem. and Cytochem.*, 30:407 (1982).

The antibodies of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147–158 (CRC Press, Inc., 1987).

Competitive binding assays rely on the ability of a labeled standard to compete with the test sample analyte for binding with a limited amount of antibody. The amount of NTNR α in the test sample is inversely proportional to the amount of standard that becomes bound to the antibodies. To facilitate determining the amount of standard that becomes bound, the antibodies generally are insolubilized before or after the competition, so that the standard and analyte that are bound to the antibodies may conveniently be separated from the standard and analyte which remain unbound.

Sandwich assays involve the use of two antibodies, each capable of binding to a different immunogenic portion, or epitope, of the protein to be detected. In a sandwich assay, the test sample analyte is bound by a first antibody which is immobilized on a solid support, and thereafter a second antibody binds to the analyte, thus forming an insoluble three-part complex. See, e.g., U.S. Pat. No. 4,376,110. The second antibody may itself be labeled with a detectable moiety (direct sandwich assays) or may be measured using an anti-immunoglobulin antibody that is labeled with a detectable moiety (indirect sandwich assay). For example, one type of sandwich assay is an ELISA assay, in which case the detectable moiety is an enzyme.

The following Examples of specific embodiments for carrying out the present invention are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

The disclosures of all publications, patents and patent applications cited herein are hereby incorporated by reference in their entirety.

EXAMPLES

Example 1

Cloning of Human NTNR α

Eight partial human cDNAs (Genbank accession numbers: R02249 (SEQ ID NO.: 7), H12981 (SEQ ID NO.: 8), W73681 (SEQ ID NO.: 9), W73633 (SEQ ID NO.: 10), H05619 (SEQ ID NO.: 11), R02135 (SEQ ID NO.: 12), T03342 (SEQ ID NO.: 13), and HSC1KA111 (SEQ ID NO.: 14)) were identified as having similarity to the GDNF

receptor a component (Jing et al. *Cell* 85:1 113–1124 (1996); Treanor et al. *Nature* 382:80–83(1996)), but were not identical to GDNFR sequences. A DNA sequence, determined by aligning these expressed-sequence-tag (“FEST”) cDNA sequences, was extended using 5' and 3' Marathon RACE reactions (Clontech Inc.) on human spleen mRNA, using conditions supplied by the manufacturer, to obtain an initial set of human cDNA clones. Additional cDNA clones were identified by screening a human fetal brain cDNA library (Stratagene) using standard protocols. Lambda cDNA libraries were plated using standard protocols and a coding region probe, obtained by PCR amplification of the NTNR α gene using the 3' and 5' RACE information, was hybridized to the library. From an alignment of the cloned human cDNA sequences, a full length cDNA sequence was obtained (SEQ ID NO: 1), which was referred to as the human Neurturin receptor a (“hNTNR α ”) cDNA sequence. This sequence contained an open reading frame sequence (SEQ ID NO: 2) that encoded a single 464 amino acid protein sequence (SEQ ID NO: 3), which was designated human Neurturin receptor α (“hNTNR α ”).

Human NTNR α (“hNTNR α ”) displays an overall 47% similarity at the amino acid level to both hGDNFR α and rGDNFR α .

hNTNR α , like hGDNFR α , is an extracellular protein that is attached to the outer cell membrane via a glycosylphosphatidyl inositol (“GPI”) modification. It has an amino terminal signal peptide for secretion, 3 potential glycosylation sites, and a stretch of 17 carboxy terminal hydrophobic amino acids preceded by a group of 3 small amino acids (Gly, Ser, Asn) defining a cleavage/binding site for GPI linkage (FIGS. 4A and 4B; Micanovic et al., *Proc. Natl. Acad. Sci. USA*, 87:157–161 (1990); Moran et al., *J. Biol. Chem.*, 266:1250–1257 (1991)). The position of their 30 cysteine residues are completely conserved between NTNR α and GDNFR α (FIGS. 5A and 5B). The extracellular domain (“ECD”) is flanked by the signal peptide and the GPI-attachment site.

Example 2

Cloning of Rat NTNR α (“rNTNR α ”)

Rat NTNR α (“rNTNR α ”) was cloned by screening a rat brain cDNA library (Clontech) using standard protocols. A full-length human NTNR α probe was hybridized to the rat cDN library at moderate stringency (e.g., 30% formamide at 42° C., wash in 0.1 \times SSC at 55° C.). The cDNA, having (SEQ ID NO: 4) and containing the complete open reading frame (SEQ ID NO: 5), was designated as rNTNR α cDNA. The open reading frame sequence encoded a single 464 amino acid protein sequence (SEQ ID NO: 6), which was designated rat Neurturin receptor α (“rNTNR α ”).

Rat NTNR α and human NTNR α display an overall 94% similarity at the amino acid level. At the DNA level, a 79% identity was observed. Rat NTNR α is 46% identical to both rat GDNFR α and human GDNFR α at the protein level. Rat NTNR α is 53% identical to rat GDNFR α at the DNA level.

rNTNR α , like rGDNFR α , hGDNFR α , and hNTNR α , is an extracellular protein that is attached to the outer cell membrane via a glycosylphosphatidyl inositol (“GPI”) modification. It has an amino terminal signal peptide for secretion, 3 potential glycosylation sites, and a stretch of 17 carboxy terminal hydrophobic amino acids preceded by a group of 3 small amino acids (Gly, Ser, Asn) defining a cleavage/binding site for GPI linkage (FIGS. 4A and 4B). Surprisingly, the position of the 30 cysteine residues, which are conserved between human and rat GDNFR α , are conserved between human and rat NTNR α (FIGS. 5A and 5B). These cysteine residues are completely conserved between NTNR α , and GDNFR α (FIGS. 5A and 5B). The extracellular domain (“ECD”) is flanked by the signal peptide and the GPI-attachment site.

Example 3

Vectors for Expression of Membrane-Bound and Soluble NTNR α

For mammalian protein expression the complete open reading frame of human NTNR α was amplified using PCR and cloned into a CMV based expression vector pRK5 or pRK7. The plasmid was designated pRK-hNTNR α for the human NTNR.

To make soluble forms of rat and human NTNR α , both rat and human NTNR α -IgG expression constructs were made by cloning the first 432 amino acids of each receptor in front of the human Fc sequence. The plasmid was designated pRK-hNTNR α -IgG for hNTNR α fusion and pRK-rNTNR α -IgG for rat NTNR α fusion. The human gene fusion coding nucleic acid sequence is SEQ ID NO: 15, which encodes the human fusion protein of SEQ ID NO: 16. The rat gene fusion coding nucleic acid sequence is SEQ ID NO: 17, which encodes the rat fusion protein of SEQ ID NO: 18.

Example 4

Tissue distribution of NTNR α .

The tissue distribution of the NTNR α mRNA was examined using in situ hybridization analysis. Its distribution was compared to that of GDNFR α .

For in situ hybridization, E15.5 rat embryos were immersion-fixed overnight at 4° C. in 4% paraformaldehyde, then cryoprotected overnight in 15% sucrose. Adult rat brains and spinal cords were frozen fresh. All tissues were sectioned at 16 μ m, and processed for in situ hybridization using ³³P-UTP labelled RNA probes as described (Davis et al. *Science* 259:1 736-1739 (1993)). Sense and antisense probes were derived from the N-terminal region of GDNFR α using T7 polymerase. NTNR α RNA was detected with a probe, derived from hNTNR α , designated hNTNR α . T7insitu probe (SEQ ID NO: 19).

In the embryonic and adult rat nervous system, mRNA for NTNR α was found in the ventral midbrain, where dopaminergic neurons are located, in parts of the ventral spinal cord where motoneurons are located, and in the dorsal root ganglia (DRG) where sensory neurons reside. In addition, high levels of NTNR α transcripts were found in tissues such as the embryonic gut, bladder, cardiac conduction system and diaphragm. In the adult rat brain NTNR α was found mainly in the substantia nigra, cortex and olfactory bulb as well as in the dorsal horn of the spinal cord. Although NTNR α was occasionally found in tissues that express GDNFR α , the two transcripts were most often present adjacent to each other. For example, in the limb, GDNFR α is expressed in muscle cells, whereas NTNR α is found in the brachial plexus nerve which enervates the muscle. Likewise, in the embryonic bladder, NTNR α is expressed in the muscle layer, while GDNFR α is expressed in the underlying epithelia. Finally, in the gut NTNR α is expressed in the mucosal epithelium, while GDNFR α is expressed only in the adjacent smooth muscles. This pattern of expression is consistent with the notion that NTNR α mediates signals inside, as well as outside, the nervous system, and suggests distinct, complementary biological roles for NTNR α and GDNFR α .

Example 5

NTNR α Specifically Binds NTN

Equilibrium binding experiments were performed to determine the binding of NTNR α to NTN. Conditioned media from 293 cells transiently transfected with either the pRK-hNTNR α -IgG or pRK-rNTNR α -IgG constructs provided soluble receptor. The conditioned media was incubated with approximately 5 pM ¹²⁵I human Neurturin, mouse Neurturin, or rGDNF along with different concen-

trations of the appropriate cold ligand in PBS containing 2 mg/ml BSA (Sigma) and 0.05% Brij 96 (Fluka) for 4 hours at room temperature. Ligand is in excess over the receptor. Receptor/ligand complexes were then incubated with protein A sepharose CL-4B (Pliarmacia) for an additional 1 hour at room temperature. After washing with PBS containing 0.2 mo/ml BSA, specific perceptible counts were measured. The IGOR program was used to determine K_d. It was found that both human and mouse ¹²⁵I-NTN can bind to recombinant soluble human NTNR α (data not shown) or soluble rNTNR α protein, specifically and reversibly with an approximate K_d of 10 pM (inset to FIG. 6C). In contrast neither human NTN (data not shown) nor mouse NTN were able to displace ¹²⁵I-rGDNF from rGDNFR α (FIG. 6B), and no high affinity binding of human or mouse ¹²⁵I-NTN to rGDNFR α was detected (FIG. 6A). Accordingly, NTN specifically binds NTNR α , interacting with high affinity with NTNR α but not with rGDNFR α . To further confirm that NTNR α is a specific receptor for NTN, competition binding experiments were performed using ¹²⁵I-rGDNF. Displaceable high affinity binding of ¹²⁵I-rGDNF to recombinant soluble rGDNFR α was readily observed (K_d of 3 pM) (FIG. 6B inset); however, binding of ¹²⁵I-rGDNF (iodinated either on tyrosine by the Bolton Hunter method or on lysine using lactoperoxidase) to either human or rat NTNR α was not detected. GDNF interacts at high affinity with GDNFR α but not with NTNR α . And, NTN specifically binds NTNR α .

Cell based equilibrium binding analysis, performed as described (Treanor et al. *Nature* 382:80-83 (1996)), confirmed the specificity of GDNFR α and NTNR α for GDNF and Neurturin, respectively. 293 cells, which were transiently transfected with either the full length NTNR α expression construct (or GDNFR α) or an irrelevant (control) construct, provided membrane-bound receptor. The observed specific associations between GDNF and GDNFR α and between NTN and NTNR α using competition binding to cells that express unmodified receptors were in agreement with the soluble receptor data (data not shown).

The ligand-binding effect of phosphoinositide-specific phospholipase C ("PIPLC") treatment of the membrane bound receptor was determined. PIPLC is an enzyme that specifically cleaves GPI linkage (Koke et al. *Prot. Express. Purification* 2:51-58(1991); Shukla, *Life Sci.*, 30:1323-1335 (1982); Rhee et al., *Science*, 244:546-550 (1989)). 293 cells, which were transiently transfected with either the full length NTNR α expression construct or an irrelevant (control) construct, were incubated with ~20,000 cpm ¹²⁵I human NTN in the presence of the indicated amounts of PIPLC for 90 minutes at room temperature (FIG. 7A). The cells were washed with ice-cold PBS containing 0.2 mg/ml BSA, after which cell associated ¹²⁵I was measured. Consistent with the prediction that NTNR α is anchored to the cell surface by a GPI linkage, the binding of ¹²⁵I-NTN to cells expressing NTNR α was significantly reduced following treatment with (FIG. 7A). Accordingly, NTNR α is a specific high affinity GPI-linked receptor for NTN.

Example 6

NTNR α Mediates Biological Response to NTN

To confirm that NTNR α mediates the biological response to NTN, the effect of NTN on the survival of cells that express NTNR α was determined. For survival assays E14 rat motoneurons were isolated, plated, and grown in triplicate wells as described. After addition of the indicated growth factors, the number of surviving neurons was determined 72 hours later (FIG. 7B). It was observed that NTN can prevent the death of primary motoneurons (FIG. 7B) that express NTNR α (as determined by in situ hybridization). Moreover, in agreement with the finding that NTN and GDNF utilize distinct receptors, quantitative differences in

the survival response of motoneurons to the two factors was detected: GDNF at saturating concentrations promoted the survival of 100% of the BDNF-responsive motoneurons; NTN, whose receptor is sparsely distributed in the embryonic ventral horn where motoneurons reside, prevented the death of only 50% of these cells (FIG. 7B).

To further confirm that NTN α is a required mediator of the NTN signal, embryonic motoneurons were treated with PIPLC and their survival in the presence of NTN or BDNF was monitored in culture. E14 rat motoneurons were isolated, plated, and grown in triplicate wells as described. PIPLC (2–4 μ g/ml) was added to the indicated samples 1–2 h prior to, as well as 12 h and 24 h following, addition of the indicated growth factors, and the number of surviving neurons was determined 72 h later (FIG. 7C). The number of embryonic rat spinal motoneurons that remained alive at saturating concentrations of NTN was reduced by 70–90% following PIPLC treatment, whereas no decrease in the response of PIPLC-treated neurons to brain-derived neurotrophic factor (BDNF) was noted. Moreover, when NTN was added to these motoneurons in combination with soluble NTN α , the response to NTN was restored (FIG. 7C). Accordingly, NTN α appears to be an essential component of the NTN signaling cascade and has the properties expected of the ligand-binding subunit of a functional NTN receptor. In addition, the soluble NTN α receptor can impart a NTN-responsiveness to cells that lack cell surface NTN α , but express the complementing Ret transmembrane protein.

Since NTN α , like GDNFR α , lacks a cytoplasmic domain and appears to be anchored to the outer surface of the cell via GPI, transmission of the NTN signals to the cell interior must involve additional proteins. As the tyrosine kinase receptor Ret, which by itself does not bind GDNF or NTN with a high affinity (Jing et al. *Cell* 85:1113–1124 (1996)); Treanor et al. *Nature* 382:80–83 (1996); data not shown), appears to be a signaling component of the GDNF receptor, we examined the possibility that it would also transduce the NTN response following binding of NTN to NTN α . To assay for tyrosine phosphorylation, cells were incubated for 1 h at 37° C. with or without PIPLC, and then exposed to various concentrations of NTN. Cells were then removed from the plates with 2 mM EDTA in PBS and lysed with ice-cold buffer (10 mM sodium phosphate (pH 7.0), 100 mM NaCl, 1% NP40, 5 mM EDTA, 100 mM sodium vanadate, 2 mM PMSF, and 0.2 units of aprotinin), and used for immunoprecipitation with antisera raised against the 19 amino acid carboxyl terminus of Ret, followed by binding to protein A sepharose. The immunoprecipitated proteins were released by boiling in SDS sample buffer, separated on an 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and reacted with anti-phosphotyrosine antibody (Upstate Biotechnology, Inc.); detection was with an ECL Western blotting detection system (Amersham Life Science). The human neuroblastoma cell line, TGW-1, which expresses endogenous c-ret (Ikeda et al. *Oncogene*

5:1291 (1990); Takahashi et al. *Oncogene* 6:297 (1991)), was exposed to NTN for 5 minutes, and the level of Ret tyrosine phosphorylation was determined. NTN clearly induced phosphorylation of Ret (FIG. 7D), as well as of the receptor tyrosine kinase responsive, cytoplasmic kinase ERK (i.e., MAPK) in this cell line (FIG. 7E), but not in 4 other neuroblastoma lines that were examined (data not shown). Furthermore, consistent with the hypothesis that NTN α is an essential mediator between NTN and Ret, NTN failed to induce significant tyrosine phosphorylation on Ret in cells that were treated with PIPLC (FIG. 7F). A similar result was obtained with GDNF.

Presented herein is a novel sequence encoding a novel receptor for neurturin, a recently discovered member of the GDNF protein family. The results presented herein reveal the existence of a novel family of multi-component receptors for growth and differentiation factors. The receptor complex is composed of a shared signaling subunit—the transmembrane tyrosine kinase receptor Ret—and a GPI-linked, receptor-specific-ligand-binding subunit—NTN α in the case of NTN, and GDNFR α in the case of GDNF. In view of these findings, further details on the mechanism of action of NTN and GDNF on the molecular level can be determined. In addition, it is now clear that the distinct biological activities of the GDNF/NTN protein family are determined by the distinct tissue distribution of their respective ligand-binding-receptor components rather than by the ability to activate different signaling systems. Finally, the data presented herein provides a contrasting and simple biological rationale for the involvement of what previously appeared to be a needlessly complex way to activate a tyrosine kinase receptor (reviewed in Lindsay and Yancopoulos, *Neuron* 17:571–574 (1996)). It is now apparent that changing the ligand-binding accessory molecule is a most economic way to recruit the same signalling system for usage by multiple growth factors, and that GPI-linked proteins are economic ligand-binding accessory molecules. Use of a single transmembrane signalling system by multiple growth factors appears to be employed by cytokines (Stahl and Yancopoulos, *Ann. Rev. Biophys. Biomol. Struct.* 24:269–291 (1993)) as well as by members of the transforming growth factor protein family (Wrana et al., *Nature* 370:341–347 (1994)). Likewise, GPI-linked proteins are used as ligand-binding accessory molecules in several multi-component receptors, such as the bacterial endotoxin receptor (Lee et al., *Proc. Natl. Acad. Sci. USA* 90:9930–9934 (1993); Pugin et al., *Proc. Natl. Acad. Sci. USA* 90:2744–2748 (1993)) and the receptor for ciliary neurotrophic factor (Davies et al., *Science* 259:1736–1739 (1993)). The discovery of the receptor and associated receptor system presented herein defines a novel paradigm in signal transduction, highlights the diverse strategies which are used to transmit extracellular signals in the vertebrate nervous system, and provides means for modulating and controlling cell activity and survival that expand the treatment modalities available to the clinician.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 28

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 2600 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCGAGAGCTG CGGGGGGAGG AGGAGGAGGG TGCCGACGCT TGAGTGGGTT	50
CGAGCCCGAG CCGTAGCCGG GGGAGCCAGT CAGTTTCCGG CCAAGGCAGC	100
AGGGAGAAAG ACAAAAAAAC GGTGGGATTT ATTTAACATG ATCTTGCAA	150
ACGTCTTCTT CCTCTTCTTC TTTCTAGACG AGACCCTCCG CTCTTTGGCC	200
AGCCCTTCCT CCCTGCAGGA CCCCGAGCTC CACGGCTGGC GCCCCCAGT	250
GGACTGTGTC CGGGCCAATG AGCTGTGTGC CGCCGAATCC AACTGCAGCT	300
CTCGCTACCG CACTCTGCGG CAGTGCTGG CAGGCCGCGA CCGCAACACC	350
ATGCTGGCCA ACAAGGAGTG CCAGGCGGCC TTGGAGGTCT TGCAGGAGAG	400
CCCGCTGTAC GACTGCCGCT GCAAGCGGGG CATGAAGAAG GAGCTGCAGT	450
GTCTGCAGAT CTA CTGGAGC ATCCACCTGG GGCTGACCGA GGGTGAGGAG	500
TTCTACGAAG CCTCCCCCTA TGAGCCGGTG ACCTCCCGCC TCTCGGACAT	550
CTTCAGGCTT GCTTCAATCT TCTCAGGGAC AGGGGCAGAC CCGGTGGTCA	600
GCGCCAAGAG CAACCATTCG CTGGATGCTG CCAAGGCCTG CAACCTGAAT	650
GACAAC TGCA AGAAGCTGCG CTCTCTCTAC ATCTCCATCT GCAACCGCGA	700
GATCTCGCCC ACCGAGCGCT GCAACCGCCG CAAGTGCCAC AAGGCCCTGC	750
GCCAGTTCTT CGACCGGGTG CCCAGCGAGT ACACCTACCG CATGCTCTTC	800
TGCTCCTGCC AAGACCAGGC GTGCGCTGAG CGCCGCCGGC AAACCATCCT	850
GCCCAGCTGC TCCTATGAGG ACAAGGAGAA GCCCAACTGC CTGGACCTGC	900
GTGGCGTGTG CCGGACTGAC CACCTGTGTC GGTCCCGGCT GGCCGACTTC	950
CATGCCAATT GTCGAGCCTC CTACCAGACG GTCACCAGCT GCCCTGCGGA	1000
CAATTACCAG GCGTGTCTGG GCTCTTATGC TGGCATGATT GGGTTTGACA	1050
TGACACCTAA CTATGTGGAC TCCAGCCCCA CTGGCATCGT GGTGTCCCCC	1100
TGGTGCAGCT GTCGTGGCAG CGGGAACATG GAGGAGGAGT GTGAGAAGTT	1150
CCTCAGGGAC TTCACCGAGA ACCCATGCCT CCGGAACGCC ATCCAGGCCT	1200
TTGGCAACGG CACGGACGTG AACGTGTCCC CAAAAGGCC CTCTGTTCCAG	1250
GCCACCCAGG CCCCTCGGGT GGAGAAGACG CCTTCTTTGC CAGATGACCT	1300
CAGTGACAGT ACCAGCTTGG GGACCAGTGT CATCACCACC TGCACGTCTG	1350
TCCAGGAGCA GGGGCTGAAG GCCAACAACT CCAAAGAGTT AAGCATGTGC	1400
TTACAGAGC TCACGACAAA TATCATCCCA GGGAGTAACA AGGTGATCAA	1450
ACCTAACTCA GGCCCCAGCA GAGCCAGACC GTCGGCTGCC TTGACCGTGC	1500
TGTCTGTCTT GATGCTGAAA CTGGCCTTGT AGGCTGTGGG AACCGAGTCA	1550
GAATATTTTT GAAAGCTACG CAGACAAGAA CAGCCGCCTG ACGAAATGGA	1600
AACACACACA GACACACACA CACCTTGCAA AAAAAAATT GTTTTTCCCA	1650
CCTTGTCGCT GAACCTGTCT CCTCCAGGT TTCTTCTCTG GAGAAGTTTT	1700
TGTAAACCAA ACAGACAAGC AGGCAGGCAG CCTGAGAGCT GGCCAGGGG	1750
TCCCCTGGCA GGGGAAACTC TGGTGCCGGG GAGGGCACGA GGCTCTAGAA	1800

-continued

ATGCCCTTCA	CTTTCTCCTG	GTGTTTTTCT	CTCTGGACCC	TTCTGAAGCA	1850
GAGACCGGAC	AAGAGCCTGC	AGCGGAAGGG	ACTCTGGGCT	GTGCCTGAGG	1900
CTGGCTGGGG	GCAGGACAAC	ACAGCTGCTT	CCCCAGGCTG	CCCCTCTGG	1950
GGACCCGCTG	GGGGCTGGCA	GAGGGCATCG	GTCAGCGGGG	CAGCGGGGCT	2000
GGCCATGAGG	GTCCACCTTC	AGCCCTTTGG	CTTCAAGGAT	GGAGATGGTT	2050
TTGCCCTCCC	TCTCTGCCCT	CGGGTGGGGC	TGGTGGGTCT	GCAGCTGGTG	2100
TGGGAACTTC	CCCACGGATG	GCGGTGGAGG	GGGTTCGCAC	CGTGCTGGGC	2150
TCCCCCTGAC	TGTAGCACGG	AGTGTGGGG	CTGGGGGCCA	GCTCCAGGAG	2200
GGCTTGAGAG	CTCAGCCTGC	CTGGGAGAGC	CCTTGTGGCG	AGGCATTAAA	2250
ACTTGGGCAC	CAGCTTCTTT	CTCGGTGGCA	GAAATTTTGA	AGTCAGAGAG	2300
AAACGGTCCT	TTGTTGGCTT	CTTTGCTTTC	TCGTGGGTCC	TTTGGCAGGC	2350
CTCCCTTTGG	GGAGAGGGAG	GGGAGAGACC	ACAGCCGGGT	GTGTGTCTGC	2400
AGCACCGTGG	GCCCTCAAGC	TTTCTGCTG	TCTTCTCCCT	CCTCCTCCTT	2450
TCCCCTTTCT	CTTTCCTCAT	TTCTTAGACG	TACGTCAACT	GTATGTACAT	2500
ACCGGGGCTC	CTCTCCTAAC	ATATATGTAT	ATACACATCC	ATATACATAT	2550
ATTGTGTGGT	TTCCCCTTTC	TTTCTTTTTT	TAAGCAACAA	AACTATGGGG	2600

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1392 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGATCTTGG	CAAACGTCTT	CTTCTCTTTC	TTCTTTCTAG	ACGAGACCCT	50
CCGCTCTTTG	GCCAGCCCTT	CCTCCCTGCA	GGACCCCGAG	CTCCACGGCT	100
GGCGCCCCC	AGTGGACTGT	GTCCGGGCCA	ATGAGCTGTG	TGCCGCCGAA	150
TCCAAC TGCA	GCTCTCGCTA	CCGCACTCTG	CGGCAGTGCC	TGGCAGGCCG	200
CGACCGCAAC	ACCATGCTGG	CCAACAAGGA	GTGCCAGGCG	GCCTTGGAGG	250
TCTTGCAAGGA	GAGCCCGCTG	TACGACTGCC	GCTGCAAGCG	GGGCATGAAG	300
AAGGAGCTGC	AGTGTCTGCA	GATCTACTGG	AGCATCCACC	TGGGGCTGAC	350
CGAGGGTGAG	GAGTCTACG	AAGCCTCCCC	CTATGAGCCG	GTGACCTCCC	400
GCCTCTCGGA	CATCTTCAGG	CTTGCTTCAA	TCTTCTCAGG	GACAGGGGCA	450
GACCCGGTGG	TCAGCGCCAA	GAGCAACCAT	TGCCTGGATG	CTGCCAAGGC	500
CTGCAACCTG	AATGACAACT	GCAAGAAGCT	GCGCTCCTCC	TACATCTCCA	550
TCTGCAACCG	CGAGATCTCG	CCCACCGAGC	GCTGCAACCG	CCGCAAGTGC	600
CACAAGGCC	TGCGCCAGTT	CTTCGACCGG	GTGCCAGCG	AGTACACCTA	650
CCGCATGCTC	TTCTGCTCCT	GCCAAGACCA	GGCGTGCCT	GAGCGCCGCC	700
GGCAAACCAT	CCTGCCAGC	TGCTCCTATG	AGGACAAGGA	GAAGCCCAAC	750
TGCCTGGACC	TGCGTGGCGT	GTGCCGGACT	GACCACCTGT	GTCGGTCCCG	800
GCTGGCCGAC	TTCCATGCCA	ATTGTCGAGC	CTCCTACCAG	ACGGTCACCA	850
GCTGCCCTGC	GGACAATTAC	CAGGCGTGTC	TGGGCTCTTA	TGCTGGCATG	900

-continued

ATTGGGTTTG ACATGACACC TAACTATGTG GACTCCAGCC CCACTGGCAT	950
CGTGGTGTCC CCCTGGTGCA GCTGTCGTGG CAGCGGGAAC ATGGAGGAGG	1000
AGTGTGAGAA GTTCCTCAGG GACTTCACCG AGAACCCATG CCTCCGGAAC	1050
GCCATCCAGG CCTTTGGCAA CGGCACGGAC GTGAACGTGT CCCCAAAAGG	1100
CCCCTCGTTC CAGGCCACCC AGGCCCTCG GGTGGAGAAG ACGCCTTCTT	1150
TGCCAGATGA CCTCAGTGAC AGTACCAGCT TGGGGACCAG TGTCATCACC	1200
ACCTGCACGT CTGTCCAGGA GCAGGGGCTG AAGGCCAACA ACTCCAAAGA	1250
GTTAAGCATG TGCTTCACAG AGCTCACGAC AAATATCATC CCAGGGAGTA	1300
ACAAGGTGAT CAAACCTAAC TCAGGCCCCA GCAGAGCCAG ACCGTCGGCT	1350
GCCTTGACCG TGCTGTCTGT CCTGATGCTG AAAGTGGCCT TG	1392

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 464 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met	Ile	Leu	Ala	Asn	Val	Phe	Phe	Leu	Phe	Phe	Phe	Leu	Asp	Glu	1	5	10	15
Thr	Leu	Arg	Ser	Leu	Ala	Ser	Pro	Ser	Ser	Leu	Gln	Asp	Pro	Glu	20	25	30	
Leu	His	Gly	Trp	Arg	Pro	Pro	Val	Asp	Cys	Val	Arg	Ala	Asn	Glu	35	40	45	
Leu	Cys	Ala	Ala	Glu	Ser	Asn	Cys	Ser	Ser	Arg	Tyr	Arg	Thr	Leu	50	55	60	
Arg	Gln	Cys	Leu	Ala	Gly	Arg	Asp	Arg	Asn	Thr	Met	Leu	Ala	Asn	65	70	75	
Lys	Glu	Cys	Gln	Ala	Ala	Leu	Glu	Val	Leu	Gln	Glu	Ser	Pro	Leu	80	85	90	
Tyr	Asp	Cys	Arg	Cys	Lys	Arg	Gly	Met	Lys	Lys	Glu	Leu	Gln	Cys	95	100	105	
Leu	Gln	Ile	Tyr	Trp	Ser	Ile	His	Leu	Gly	Leu	Thr	Glu	Gly	Glu	110	115	120	
Glu	Phe	Tyr	Glu	Ala	Ser	Pro	Tyr	Glu	Pro	Val	Thr	Ser	Arg	Leu	125	130	135	
Ser	Asp	Ile	Phe	Arg	Leu	Ala	Ser	Ile	Phe	Ser	Gly	Thr	Gly	Ala	140	145	150	
Asp	Pro	Val	Val	Ser	Ala	Lys	Ser	Asn	His	Cys	Leu	Asp	Ala	Ala	155	160	165	
Lys	Ala	Cys	Asn	Leu	Asn	Asp	Asn	Cys	Lys	Lys	Leu	Arg	Ser	Ser	170	175	180	
Tyr	Ile	Ser	Ile	Cys	Asn	Arg	Glu	Ile	Ser	Pro	Thr	Glu	Arg	Cys	185	190	195	
Asn	Arg	Arg	Lys	Cys	His	Lys	Ala	Leu	Arg	Gln	Phe	Phe	Asp	Arg	200	205	210	
Val	Pro	Ser	Glu	Tyr	Thr	Tyr	Arg	Met	Leu	Phe	Cys	Ser	Cys	Gln	215	220	225	
Asp	Gln	Ala	Cys	Ala	Glu	Arg	Arg	Arg	Gln	Thr	Ile	Leu	Pro	Ser	230	235	240	
Cys	Ser	Tyr	Glu	Asp	Lys	Glu	Lys	Pro	Asn	Cys	Leu	Asp	Leu	Arg				

-continued

	245		250		255
Gly Val Cys Arg Thr Asp His Leu Cys Arg Ser Arg Leu Ala Asp	260		265		270
Phe His Ala Asn Cys Arg Ala Ser Tyr Gln Thr Val Thr Ser Cys	275		280		285
Pro Ala Asp Asn Tyr Gln Ala Cys Leu Gly Ser Tyr Ala Gly Met	290		295		300
Ile Gly Phe Asp Met Thr Pro Asn Tyr Val Asp Ser Ser Pro Thr	305		310		315
Gly Ile Val Val Ser Pro Trp Cys Ser Cys Arg Gly Ser Gly Asn	320		325		330
Met Glu Glu Glu Cys Glu Lys Phe Leu Arg Asp Phe Thr Glu Asn	335		340		345
Pro Cys Leu Arg Asn Ala Ile Gln Ala Phe Gly Asn Gly Thr Asp	350		355		360
Val Asn Val Ser Pro Lys Gly Pro Ser Phe Gln Ala Thr Gln Ala	365		370		375
Pro Arg Val Glu Lys Thr Pro Ser Leu Pro Asp Asp Leu Ser Asp	380		385		390
Ser Thr Ser Leu Gly Thr Ser Val Ile Thr Thr Cys Thr Ser Val	395		400		405
Gln Glu Gln Gly Leu Lys Ala Asn Asn Ser Lys Glu Leu Ser Met	410		415		420
Cys Phe Thr Glu Leu Thr Thr Asn Ile Ile Pro Gly Ser Asn Lys	425		430		435
Val Ile Lys Pro Asn Ser Gly Pro Ser Arg Ala Arg Pro Ser Ala	440		445		450
Ala Leu Thr Val Leu Ser Val Leu Met Leu Lys Leu Ala Leu	455		460		464

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3358 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGGTGGCGG CCGCTCTAGA ACTAGTGGAT CCCC GGCTG CAGGAATTCG	50
GCACGAGAGT GAGCCGAGCA AGGTTAGCG GGAGAAGATT TTTTTTTTTT	100
TGAATCTTTT TCTTCGTCTT GGTGCCAAAG AAGCGACTCT GGTCTCCCGT	150
CCTAGAAGCT CCTACTGGAT TGCTCCTATT CCGTCGGTGG ATTTCTTTCC	200
TATTCGCATT TATTCTGACC CCCTCCCTCG CTGCTTCCTT CCAGCCCTTC	250
ACTTTCAGAT CGCCTCGCCC CCACCTCTCC AGTCCCCTCC TGGGAAGTGC	300
AGGGGAATG GACCCACGGG GACTCACGCC TTCCCGGACG CGCGAGCAAA	350
GGGCTGGGCT GACCTCAGGA CCAGGCTGTT GGCTTAGAAG GCAGCCAGAC	400
ACATAGCTAC GTGTGTTTGA TTCAGTGGC AAGGGGGGAC GTCGAGAGGC	450
AGCCCACCGC CCGCCTCCTA CCCCTCCCCC TCCAACCAGC AGTGAGAATC	500
CCAGGACTCG GGATCTTCAA CCGGCGGCCG CCCGGCGGGA TCTCCGCATT	550
GGATTTGGGG GTCGTTATTG CTCGGCTGTT ATTATTATCG TTATTTTATT	600

-continued

TTTATTTTTT	AAACCTAAGG	GAGAAAGACA	CATACACACA	AAACTGTGGG	650
ATTTATTTAA	CATGATCTTG	GCAAACGCCT	TCTGCCTCTT	CTTCTTTTTA	700
GACGAAACCC	TCCGCTCTTT	GGCCAGCCCT	TCCTCCCTGC	AGGGCTCTGA	750
GCTCCACGGC	TGGCGCCCC	AAGTGGACTG	TGTCCGGGCC	AATGAGCTGT	800
GTGCGGCTGA	ATCCAAGTGC	AGCTCCAGGT	ACCGCACCCCT	TCGGCAGTGC	850
CTGGCAGGCC	GGGATCGCAA	TACCATGCTG	GCCAATAAAGG	AGTGCCAGGC	900
AGCCCTGGAG	GTCTTGACAG	AAAGCCCCT	GTATGACTGC	CGCTGCAAGC	950
GGGGCATGAA	GAAGGAGCTG	CAGTGTCTGC	AGATCTACTG	GAGCATCCAT	1000
CTGGGGCTGA	CAGAGGGTGA	GGAGTTCTAT	GAAGCTTCCC	CCTATGAGCC	1050
TGTGACCTCG	CGCCTCTCGG	ACATCTTCAG	GCTCGCTTCA	ATCTTCTCAG	1100
GGACAGGGAC	AGACCCGGCG	GTCAGTACCA	AAAGCAACCA	CTGCCTGGAT	1150
GCCGCCAAGG	CCTGCAACCT	GAATGACAAC	TGCAAGAAGC	TTCGCTCCTC	1200
TTATATCTCC	ATCTGCAACC	GTGAGATCTC	TCCCACCGAA	CGCTGCAACC	1250
GCCGCAAGTG	CCACAAGGCT	CTGCGCCAGT	TCTTTGACCG	TGTGCCCAGC	1300
GAGTATACCT	ACCGCATGCT	CTTCTGCTCC	TGTCAGGACC	AGGCATGTGC	1350
TGAGCGTCGC	CGGCAAACCA	TCCTGCCCAG	TTGCTCCTAT	GAGGACAAGG	1400
AGAAGCCCAA	CTGCCTGGAC	CTGCGCAGCC	TGTGTGCTAC	AGACCACCTG	1450
TGCCGGTCCC	GACTGGCAGA	TTTCCACGCC	AACTGTGAG	CCTCCTACCG	1500
GACAATCACC	AGCTGTCCCTG	CGGACAACCTA	CCAGGCATGT	CTGGGCTCCT	1550
ATGCTGGCAT	GATTGGGTTT	GATATGACAC	CCAACTATGT	GGACTCCAAC	1600
CCCACGGGCA	TCGTGGTGTG	TCCCTGGTGC	AATTGTCGTG	GCAGTGGGAA	1650
CATGGAAGAA	GAGTGTGAGA	AGTTCCTCAG	GGACTTCACG	GAAAACCCAT	1700
GCCTCCGGAA	TGCCATTCAG	GCCTTTGGTA	ATGGCACAGA	TGTGAACATG	1750
TCTCCCAAAG	GCCCCTCACT	CCCAGCTACC	CAGGCCCTC	GGGTGGAGAA	1800
GACTCCTTCA	CTGCCAGATG	ACCTCAGTGA	CAGCACCAGC	CTGGGGACCA	1850
GTGTCATCAC	CACCTGCACA	TCTATCCAGG	AGCAAGGGCT	GAAGGCCAAC	1900
AACTCCAAAG	AGTTAAGCAT	GTGCTTCACA	GAGCTCACGA	CAAACATCAG	1950
TCCAGGGAGT	AAAAAGGTGA	TCAAACCTTAA	CTCAGGCTCC	AGCAGAGCCA	2000
GACTGTCCGC	TGCCTTGACT	GCCCTCCCAC	TCCTGATGCT	GACCTTGCC	2050
TTGTAGGCCT	TTGGAACCCA	GCACAAAAGT	TCTTCAAGCA	ACCCAGATAT	2100
GAACTCCCGC	CTGACAAAAT	GGAAACACAC	GCATACACAC	ATGCACACAC	2150
ACACAAACAC	ACACACACAC	ACACACACAC	ACACACACAC	ACACACACAC	2200
ACACCCCTTG	CAAAAACACT	TTTTTTCCTA	CATTGTCTCT	GAACCTTCT	2250
CCTCCCAAGT	TTCTTCTCTG	GAGAAGTTTT	TCTAAACCAA	ACAGACAAGC	2300
AGGCGGGCAG	TCAGAAGCCT	GCCCAGAGGT	CCCCTGCAAG	GGACACCCAG	2350
CACCAACGAG	GGCTCAAGGC	TCTTGAGAGA	CTCTTTTCTC	TTCACTGGTG	2400
TTTTCTCTCT	GGACAAGATG	AGACCCTGAT	GTGGAAGGTA	CTTTGCTGTG	2450
CCTGGTGTGG	ACTGGGGAAA	GGACAGTTGC	AGCTGCCTAC	TCTGGGGACC	2500
TGCCCAAGGG	TTCACAGAGA	GTCTCAGTCA	GCAAGGAAGC	AGGGCTGGCC	2550
ACAAGGACTT	TGTCACCTCT	TCCTCTTGGC	TTCAAGAGATG	GAAATGGTTT	2600

-continued

GCTGCCATCC CCAGCCATTA TGTGGCCTAG TGGGTTTAAAG TCTGGAGTAG	2650
GAAGCCTCAT GGCAGCTTCA GGCCATGGTG CCTGTAGTAT AGCTGGGGTT	2700
GGGAGCTGTT ACAGGAGGAA GCTTCTTTGG GGCATGAGCA AGCCTTGTT	2750
GGGCACCAGC TCCAAGATGT ACCTTCCTCC TTTATGCCAG GAATCTTGAA	2800
GTCAAAGAGA AATGATCCTC TGTGGCTCT TTTTGTGTTG TTTTGAATT	2850
TTTTTGTGGG TCATTGTC AGGTCTCTCT TGGGGAGAAG GGCTGTTGAG	2900
TGGGGCTGGG GAGACCCTAG CTGGGCGTGT GTATGGAGCA CTCTGGTGGG	2950
TTCCCAAGCT TGCCCTTCT CTCTTCTTGT TTCTGCTTTC TCTCTCATTT	3000
CTGAGACATT CATGCACTGT CTGTACATAC TGGGTCTCCT TTCTCAACAT	3050
ATGTGTATAT CCATATCCAT ATATCCTATG ATTTTACTCT TTCTTTCATT	3100
TTTTTTTAAAG AAACAAAAC ATGGAATAA TACCCTACAG ATGAGCGAAA	3150
ATGTATTATT GTAAAGTTTA TTTTTTTTAA TAATGTTGTC TATGATGGGA	3200
AGAAAGGTAC CAGGACCCCT GAGCCCTGGT CCAGTTGGGC TGGTGGGGCT	3250
GTGGCCGGGG ACTCCCGATT GCATTCCTC TTAACCAAGC TCCAATAAAC	3300
GTACTAGGAA GCGAAAAAA AAAAAAAAAA ACTCGAGGGG GGGCCCGGTA	3350
CCCAATTC	3358

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1392 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATGATCTTGG CAAACGCCTT CTGCCTCTTC TTCTTTTTAG ACGAAACCCT	50
CCGCTCTTTG GCCAGCCCTT CCTCCCTGCA GGGCTCTGAG CTCCACGGCT	100
GGCGCCCCCA AGTGGACTGT GTCCGGGCCA ATGAGCTGTG TGCGGCTGAA	150
TCCAAC TGCA GCTCCAGGTA CCGCACCTT CGGCAGTGCC TGGCAGGCCG	200
GGATCGCAAT ACCATGCTGG CCAATAAGGA GTGCCAGGCA GCCCTGGAGG	250
TCTTGCAGGA AAGCCACTG TATGACTGCC GCTGCAAGCG GGGCATGAAG	300
AAGGAGCTGC AGTGTCTGCA GATCTACTGG AGCATCCATC TGGGGCTGAC	350
AGAGGGTGAG GAGTTCATG AAGCTTCCCC CTATGAGCCT GTGACCTCGC	400
GCCTCTCGGA CATCTTCAGG CTCGCTTCAA TCTTCTCAGG GACAGGGACA	450
GACCCGGCGG TCAGTACCAA AAGCAACCAC TGCCTGGATG CCGCCAAGGC	500
CTGCAACCTG AATGACAAC GCAAGAAGCT TCGCTCCTCT TATATCTCCA	550
TCTGCAACCG TGAGATCTCT CCCACCGAAC GCTGCAACCG CCGCAAGTGC	600
CACAAGGCTC TCGCCAGTT CTTTGACCGT GTGCCAGCG AGTATACCTA	650
CCGCATGCTC TTCTGCTCCT GTCAGGACCA GGCATGTGCT GAGCGTCGCC	700
GGCAAACCAT CCTGCCAGT TGCTCCTATG AGGACAAGGA GAAGCCCAAC	750
TGCCTGGACC TCGCAGCCT GTGTCGTACA GACCACCTGT GCCGGTCCCG	800
ACTGGCAGAT TTCCACGCCA ACTGTCGAGC CTCCTACCGG ACAATCACCA	850
GCTGTCCTGC GGACAACCTAC CAGGCATGTC TGGGCTCCTA TGCTGGCATG	900

-continued

ATTGGGTTTG ATATGACACC CAACTATGTG GACTCCAACC CCACGGGCAT 950
 CGTGGTGTCT CCCTGGTGCA ATTGTCGTGG CAGTGGGAAC ATGGAAGAAG 1000
 AGTGTGAGAA GTTCCTCAGG GACTTCACGG AAAACCCATG CCTCCGGAAT 1050
 GCCATTGAGG CCTTTGGTAA TGGCACAGAT GTGAACATGT CTCCCAAAGG 1100
 CCCCTCACTC CCAGCTACCC AGGCCCTCG GGTGGAGAAG ACTCCTTCAC 1150
 TGCCAGATGA CCTCAGTGAC AGCACCAGCC TGGGGACCAG TGTCATCACC 1200
 ACCTGCACAT CTATCCAGGA GCAAGGGCTG AAGGCCAACA ACTCCAAAGA 1250
 GTTAAGCATG TGCTTCACAG AGCTCACGAC AAACATCAGT CCAGGGAGTA 1300
 AAAAGGTGAT CAAACTTAAC TCAGGCTCCA GCAGAGCCAG ACTGTGCGCT 1350
 GCCTTGACTG CCCTCCCACT CCTGATGCTG ACCTTGGCCT TG 1392

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 464 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Ile Leu Ala Asn Ala Phe Cys Leu Phe Phe Phe Leu Asp Glu
 1 5 10 15
 Thr Leu Arg Ser Leu Ala Ser Pro Ser Ser Leu Gln Gly Ser Glu
 20 25 30
 Leu His Gly Trp Arg Pro Gln Val Asp Cys Val Arg Ala Asn Glu
 35 40 45
 Leu Cys Ala Ala Glu Ser Asn Cys Ser Ser Arg Tyr Arg Thr Leu
 50 55 60
 Arg Gln Cys Leu Ala Gly Arg Asp Arg Asn Thr Met Leu Ala Asn
 65 70 75
 Lys Glu Cys Gln Ala Ala Leu Glu Val Leu Gln Glu Ser Pro Leu
 80 85 90
 Tyr Asp Cys Arg Cys Lys Arg Gly Met Lys Lys Glu Leu Gln Cys
 95 100 105
 Leu Gln Ile Tyr Trp Ser Ile His Leu Gly Leu Thr Glu Gly Glu
 110 115 120
 Glu Phe Tyr Glu Ala Ser Pro Tyr Glu Pro Val Thr Ser Arg Leu
 125 130 135
 Ser Asp Ile Phe Arg Leu Ala Ser Ile Phe Ser Gly Thr Gly Thr
 140 145 150
 Asp Pro Ala Val Ser Thr Lys Ser Asn His Cys Leu Asp Ala Ala
 155 160 165
 Lys Ala Cys Asn Leu Asn Asp Asn Cys Lys Lys Leu Arg Ser Ser
 170 175 180
 Tyr Ile Ser Ile Cys Asn Arg Glu Ile Ser Pro Thr Glu Arg Cys
 185 190 195
 Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe Phe Asp Arg
 200 205 210
 Val Pro Ser Glu Tyr Thr Tyr Arg Met Leu Phe Cys Ser Cys Gln
 215 220 225
 Asp Gln Ala Cys Ala Glu Arg Arg Arg Gln Thr Ile Leu Pro Ser
 230 235 240

-continued

Cys	Ser	Tyr	Glu	Asp	Lys	Glu	Lys	Pro	Asn	Cys	Leu	Asp	Leu	Arg
				245					250					255
Ser	Leu	Cys	Arg	Thr	Asp	His	Leu	Cys	Arg	Ser	Arg	Leu	Ala	Asp
				260					265					270
Phe	His	Ala	Asn	Cys	Arg	Ala	Ser	Tyr	Arg	Thr	Ile	Thr	Ser	Cys
				275					280					285
Pro	Ala	Asp	Asn	Tyr	Gln	Ala	Cys	Leu	Gly	Ser	Tyr	Ala	Gly	Met
				290					295					300
Ile	Gly	Phe	Asp	Met	Thr	Pro	Asn	Tyr	Val	Asp	Ser	Asn	Pro	Thr
				305					310					315
Gly	Ile	Val	Val	Ser	Pro	Trp	Cys	Asn	Cys	Arg	Gly	Ser	Gly	Asn
				320					325					330
Met	Glu	Glu	Glu	Cys	Glu	Lys	Phe	Leu	Arg	Asp	Phe	Thr	Glu	Asn
				335					340					345
Pro	Cys	Leu	Arg	Asn	Ala	Ile	Gln	Ala	Phe	Gly	Asn	Gly	Thr	Asp
				350					355					360
Val	Asn	Met	Ser	Pro	Lys	Gly	Pro	Ser	Leu	Pro	Ala	Thr	Gln	Ala
				365					370					375
Pro	Arg	Val	Glu	Lys	Thr	Pro	Ser	Leu	Pro	Asp	Asp	Leu	Ser	Asp
				380					385					390
Ser	Thr	Ser	Leu	Gly	Thr	Ser	Val	Ile	Thr	Thr	Cys	Thr	Ser	Ile
				395					400					405
Gln	Glu	Gln	Gly	Leu	Lys	Ala	Asn	Asn	Ser	Lys	Glu	Leu	Ser	Met
				410					415					420
Cys	Phe	Thr	Glu	Leu	Thr	Thr	Asn	Ile	Ser	Pro	Gly	Ser	Lys	Lys
				425					430					435
Val	Ile	Lys	Leu	Asn	Ser	Gly	Ser	Ser	Arg	Ala	Arg	Leu	Ser	Ala
				440					445					450
Ala	Leu	Thr	Ala	Leu	Pro	Leu	Leu	Met	Leu	Thr	Leu	Ala	Leu	
				455					460				464	

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 229 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCAAGAGCAA CCATTGCCTG GATGCTGCCA AGGCCTGCAA CCTGAATGAC	50
AACTGCAAGA AGCTGCGCTC CTCCTACATC TCCATCTGCA ACCGCGAGAT	100
CTCGCCACC GAGCGCTGCA ACCGCCGCAA GTGCCACAAG GCCCTGCGCC	150
AGTTCTTCGA CCGGGTGCCC AGCGAGTACA CCTACCGCAT GCTCTTCTGC	200
TCCTGCCAAG ATCAGGCGTG CGCTGAGNC	229

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 521 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAACCATTGC CTGGGATGCT GCCAAGGCCT GCAACCTGAA TGACAACTGC	50
--	----

-continued

AAGAAGCTGC GCTCCTCCTA CATCTCCATC TGCAACCGCG AGATCTCGCC	100
CACCGAGCGC TGCAACCGCC GCAAGTGCCA CAAGGCCCTG CGCCAGTTCT	150
TCGACCGGGT GCCCAGCGAG TACACCTACC GCATGCTCTT CTGCTCCTGC	200
CAAGACCAGG CGTGCCTGA GCCCGGNCA AAACCATCCT GCCCAGCTGC	250
TCCTATGAGG ACAAGGAGAA GCCCAACTGC CTGGGACCTG CGTGGCGTGT	300
GCCGGGACTG ACCACCTGTG TCGGTCCCGG CTNGGCCGAC TTTCCATGGC	350
CAATTTGTTG GAGCCTTCCT ACCAGACGGG TCANCAGGTT GCCTTGCGGA	400
CAATTTACCA GGGGTNTTTT GGGGTTTTTA TTGTTGGGCA TGGATTGGGG	450
TTTTGAAATT GANAATTAAT TTTGTTGGGA TTTNCAGGCC CCATTGGGCN	500
TTGTNGGTGN TTCCCCTGGG G	521

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 478 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGACACCTAA CTATGTGGAC TCCAGCCCCA CTGGCATCGT GGTGTCCCCC	50
TGGTGCAGCT GTCGTGGCAG CGGGAACATG GAGGAGGAGT GTGAGAAGTT	100
CCTCAGGGAC TTCACCGAGA ACCCATGCCT CCGGAACGCC ATCCAGGCCT	150
TTGNAACGGC ACGGACGTGA ACGTGTCCCC AAAAGGCCCC TCGTTCAGG	200
CCACCCAGGC CCTCGGGTGG AGAAGACGCC TTCTTTGCCA GATGACCTCA	250
GTGACAGTAC CAGCTTGGGG ACCAGTGTC TACCACCTG CACGTCTGTC	300
CAGGAGCAGG GGCTGAAGGC CAACAACCTC AAAGAGTTAA GCATGTGCTT	350
CACAGAGCTC ACCGACAAAT ATCATCCCAG GGAGTAACAA GGTGATTCAA	400
ACCTAACTCA GGCCCCAGCA GAGCAAGACC GTCGGCTTGC CTTTGACCGT	450
GCTGTCTGTC CTGATGCTGA ACAGGCTT	478

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 433 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCAAGGTGTG TGTGTGTCTG TGTGTGTTT CATTTCGTCA GGCGGCTGTT	50
CTTGTCTGCG TACTTTCAA AATCTTCTGA CTCGGTTCCC ACAGCCTACA	100
AGGCCTGTTT CAGCATCAGG ACAGACAGCA CGGTCAAGGC AGCCGACGGT	150
CTGGCTCTGC TGGGGCCTGA GTTAGGTTT ATCACCTTGT TACTCCCTGG	200
GATGATATTT GTCGTGAGCT CTGTGAAGCA CATGCTTAAC TCTTTGGAGT	250
TGTTGGCCTT CAGCCCCTGC TCCTGGACAG ACGTGCAGGT GGTGATGACA	300
CTGGGTCCCC AAGCTGGTAC TGTCACCTGAG GTCATCTGGC AAAGAAGGCG	350
TCTTCTCCAC CCGAGGGGCC TGGGGTGGCT GGGAACGAGG GGGCCTTTTT	400
GGGGGACACG TTCACGTTCC GTTGCCGTTG CCA	433

-continued

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 418 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

TTTTTTTTTT TGGGAAAAAC AATTTTTTTT TTGCAAGGTG TGTGTGTGTC      50
TGTGTGTGTT TCCATTTTCGT CAGGCGGCTG TTCTTGTCTG CGTANTTTTC      100
AAAAATCTTC TGACTCGGTT CCCACAGCCT ACAAGGCCTG TTTCAGCATC      150
AGGACAGACA GCACGGTCAA GGCAGCCGAC GGTCTGGCTC TGCTGGGGCC      200
TGAGTTAGGT TTGATCACCT TGTACTCCC TGGGATGATA TTTNTCGTGA      250
GCTCTGTGAA GCACATGCTT AACTCTTTGG AGTTNTTGGC CTTAGCCCC      300
TGCTCCTGGG ACAGAACGTG CAGGNTGGGT GATGACACTG GGNCCCCAAG      350
GCTGGGTACT GTCACTGAGG GTCATCTGGN CAAAGNAAGG NCGTTTTTCT      400
CCACCCGAGG GGCCGGGG      418

```

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 364 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

TGTGTGTGTC TGTGTGTGTT TCCATTTTCGT CAGGCGGCTG TTCTTGTCTG      50
CGTAGTTTCA AAAATCTTCT GACTCGGTTT CCACAGCCTA CAAGGNCTGT      100
TTCAGCATCA GGACAGACAG CACGGTCAAG GCAGCCGACG GTCTGGCTCT      150
GCTGGGGCCT GAGTTAGGTT TGATCACCTT GTTACTCCCT GGGATGATAT      200
TTGTCGTGAG CTCTGTGAAG CACATGCTTA ACTCTTTGGA GTTGTGGCC      250
TTCAGCCCCT GTCCTGGAC AGACGTGCAG GTGGTNATGA CACTGGTCCC      300
CAAGCTGGTA CTNTCACTGA GTCATCTGG CAAAGAAGGC GTCTTCTCCA      350
CCCNAGGGGC CTGG      364

```

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 319 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

GGGAAAAACA ATTTTATTTT TGCAAGGTGT GTGTGTGTCT GTGTGTGTTT      50
CCATTTTCGTC AGGCGGCTGT CCTTGTCTGC GTAGTTTCAA AAATCTTCTG      100
ACTCGGTTCC CACAGCCTAC AAGGCCTGTA TAAGCATCAG GACAGACAGC      150
ACGGTCAAGG CAGCCGACGG TCTGGCTCTG CTGGGGCCTG AGTAAGGTTT      200
GNCCACCTTG TAACTCCCTG GGATGATATT TGTCGTGAGC NCTGTNANGC      250
ACATGNTTAA CTCTTTGGAG TTNTTGGCCT TCAGCCCCTG CCCCTGGNCA      300

```


-continued

GACGTGCAGG TGGTGATGA 319

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 309 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCTGAAACTG GCCTTGTAGG CTGTGGGAAC CGAGTCAGAA TATTTTTGAA 50
 AGCTACGCAG ACAAGAACNG CGGCCTGACG AAATGGAAAC ACACACAGAC 100
 ACACACACNC CTTGCATAAA AAAAAATTGTT TTTCCACCT TGTCGCTGAA 150
 CCTGTCTCCT CCCAGGTTTC TTCTCTGGAG AAGTTTTTGT AAACCAAACA 200
 GACAAGCAGG CAGGCAGCCT GAGAGCTGGC CCAGGGGTCC CCTGGTCAGG 250
 GGAAACTCTG GTGCCGGGGA GGGCACGTGG CTCTAGAAAT GCCCTTCACT 300
 TTCTCCTGG 309

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1995 base pairs
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATGATCTTGG CAAACGTCTT CTTCTCTTC TTCTTTCTAG ACGAGACCCT 50
 CCGCTCTTTG GCCAGCCCTT CCTCCCTGCA GGACCCCGAG CTCCACGGCT 100
 GCGCCCCC AGTGGACTGT GTCCGGGCA ATGAGCTGTG TGCCGCCGAA 150
 TCCAAGTCA GCTCTCGTA CCGACTCTG CGGCAGTGCC TGGCAGGCCG 200
 CGACCGCAAC ACCATGCTGG CCAACAAGGA GTGCCAGGCG GCCTTGGAGG 250
 TCTTGCAGGA GAGCCCGCTG TACGACTGCC GCTGCAAGCG GGGCATGAAG 300
 AAGGAGCTGC AGTGTCTGCA GATCTACTGG AGCATCCACC TGGGGCTGAC 350
 CGAGGGTGAG GAGTTCTACG AAGCCTCCCC CTATGAGCCG GTGACCTCCC 400
 GCCTCTCGGA CATCTTCAGG CTTGCTTCAA TCTTCTCAGG GACAGGGGCA 450
 GACCCGGTGG TCAGCGCAA GAGCAACCAT TGCCTGGATG CTGCCAAGGC 500
 CTGCAACCTG AATGACAAC TCAAGAAGCT GCGCTCCTCC TACATCTCCA 550
 TCTGCAACCG CGAGATCTCG CCCACCGAGC GCTGCAACCG CCGCAAGTGC 600
 CACAAGGCC TGCGCCAGTT CTTGACCGG GTGCCAGCG AGTACACCTA 650
 CCGCATGCTC TTCTGCTCCT GCCAAGACCA GCGGTGCGCT GAGCGCCGCC 700
 GGCAAACCAT CCTGCCAGC TGCTCCTATG AGGACAAGGA GAAGCCCAAC 750
 TGCCTGGACC TGCCTGGCGT GTGCCGACT GACCACCTGT GTCGGTCCCG 800
 GCTGGCCGAC TTCCATGCCA ATTGTCGAGC CTCCTACCAG ACGGTCACCA 850
 GCTGCCCTGC GGACAATTAC CAGGCGTGTG TGGGCTCTTA TGCTGGCATG 900
 ATTGGGTTT ACATGACACC TAACTATGTG GACTCCAGCC CCACTGGCAT 950
 CGTGGTGTCC CCCTGGTGCA GCTGTCGTGG CAGCGGGAAC ATGGAGGAGG 1000

-continued

AGTGTGAGAA GTTCCTCAGG GACTTCACCG AGAACCCATG CCTCCGGAAC	1050
GCCATCCAGG CCTTTGGCAA CGGCACGGAC GTGAACGTGT CCCCAAAAGG	1100
CCCCTCGTTC CAGGCCACCC AGGCCCTCG GGTGGAGAAG ACGCCTTCTT	1150
TGCCAGATGA CCTCAGTGAC AGTACCAGCT TGGGGACCAG TGTCATCACC	1200
ACCTGCACGT CTGTCCAGGA GCAGGGGCTG AAGGCCAACA ACTCCAAAGA	1250
GTTAAGCATG TGCTTCACAG AGCTCACGAC AAATATCATC CCAGGGCCTA	1300
GGGACCCGGT GGACAAAAC CACACATGCC CACCGTGCCC AGCACCTGAA	1350
CTCCTGGGGG GACCGTCAGT CTTCTCTTC CCCCCAAAAC CCAAGGACAC	1400
CCTCATGATC TCCCGGACCC CTGAGGTCAC ATGCGTGGTG GTGGACGTGA	1450
GCCACGAAGA CCCTGAGGTC AAGTTCAACT GGTACGTGGA CGGCGTGGAG	1500
GTGCATAATG CCAAGACAAA GCCGCGGGAG GAGCAGTACA ACAGCACGTA	1550
CCGTGTGGTC AGCGTCCTCA CCGTCCTGCA CCAGGACTGG CTGAATGGCA	1600
AGGAGTACAA GTGCAAGGTC TCCAACAAAG CCCTCCCAGC CCCCATCGAG	1650
AAAACCATCT CCAAAGCCAA AGGGCAGCCC CGAGAACCAC AGGTGTACAC	1700
CCTGCCCCCA TCCCGGGAAG AGATGACCAA GAACCAGGTC AGCCTGACCT	1750
GCCTGGTCAA AGGCTTCTAT CCCAGCGACA TCGCCGTGGA GTGGGAGAGC	1800
AATGGGCAGC CGGAGAACAA CTACAAGACC ACGCCTCCCG TGCTGGACTC	1850
CGACGGCTCC TTCTTCTCT ACAGCAAGCT CACCGTGGAC AAGAGCAGGT	1900
GGCAGCAGGG GAACGTCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC	1950
AACCACTACA CGCAGAAGAG CCTCTCCCTG TCTCCGGGTA AATGA	1995

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 664 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Met Ile Leu Ala Asn Val Phe Phe Leu Phe Phe Phe Leu Asp Glu	
1 5 10 15	
Thr Leu Arg Ser Leu Ala Ser Pro Ser Ser Leu Gln Asp Pro Glu	
20 25 30	
Leu His Gly Trp Arg Pro Pro Val Asp Cys Val Arg Ala Asn Glu	
35 40 45	
Leu Cys Ala Ala Glu Ser Asn Cys Ser Ser Arg Tyr Arg Thr Leu	
50 55 60	
Arg Gln Cys Leu Ala Gly Arg Asp Arg Asn Thr Met Leu Ala Asn	
65 70 75	
Lys Glu Cys Gln Ala Ala Leu Glu Val Leu Gln Glu Ser Pro Leu	
80 85 90	
Tyr Asp Cys Arg Cys Lys Arg Gly Met Lys Lys Glu Leu Gln Cys	
95 100 105	
Leu Gln Ile Tyr Trp Ser Ile His Leu Gly Leu Thr Glu Gly Glu	
110 115 120	
Glu Phe Tyr Glu Ala Ser Pro Tyr Glu Pro Val Thr Ser Arg Leu	
125 130 135	
Ser Asp Ile Phe Arg Leu Ala Ser Ile Phe Ser Gly Thr Gly Ala	
140 145 150	

-continued

Asp	Pro	Val	Val	Ser	Ala	Lys	Ser	Asn	His	Cys	Leu	Asp	Ala	Ala	155	160	165
Lys	Ala	Cys	Asn	Leu	Asn	Asp	Asn	Cys	Lys	Lys	Leu	Arg	Ser	Ser	170	175	180
Tyr	Ile	Ser	Ile	Cys	Asn	Arg	Glu	Ile	Ser	Pro	Thr	Glu	Arg	Cys	185	190	195
Asn	Arg	Arg	Lys	Cys	His	Lys	Ala	Leu	Arg	Gln	Phe	Phe	Asp	Arg	200	205	210
Val	Pro	Ser	Glu	Tyr	Thr	Tyr	Arg	Met	Leu	Phe	Cys	Ser	Cys	Gln	215	220	225
Asp	Gln	Ala	Cys	Ala	Glu	Arg	Arg	Arg	Gln	Thr	Ile	Leu	Pro	Ser	230	235	240
Cys	Ser	Tyr	Glu	Asp	Lys	Glu	Lys	Pro	Asn	Cys	Leu	Asp	Leu	Arg	245	250	255
Gly	Val	Cys	Arg	Thr	Asp	His	Leu	Cys	Arg	Ser	Arg	Leu	Ala	Asp	260	265	270
Phe	His	Ala	Asn	Cys	Arg	Ala	Ser	Tyr	Gln	Thr	Val	Thr	Ser	Cys	275	280	285
Pro	Ala	Asp	Asn	Tyr	Gln	Ala	Cys	Leu	Gly	Ser	Tyr	Ala	Gly	Met	290	295	300
Ile	Gly	Phe	Asp	Met	Thr	Pro	Asn	Tyr	Val	Asp	Ser	Ser	Pro	Thr	305	310	315
Gly	Ile	Val	Val	Ser	Pro	Trp	Cys	Ser	Cys	Arg	Gly	Ser	Gly	Asn	320	325	330
Met	Glu	Glu	Glu	Cys	Glu	Lys	Phe	Leu	Arg	Asp	Phe	Thr	Glu	Asn	335	340	345
Pro	Cys	Leu	Arg	Asn	Ala	Ile	Gln	Ala	Phe	Gly	Asn	Gly	Thr	Asp	350	355	360
Val	Asn	Val	Ser	Pro	Lys	Gly	Pro	Ser	Phe	Gln	Ala	Thr	Gln	Ala	365	370	375
Pro	Arg	Val	Glu	Lys	Thr	Pro	Ser	Leu	Pro	Asp	Asp	Leu	Ser	Asp	380	385	390
Ser	Thr	Ser	Leu	Gly	Thr	Ser	Val	Ile	Thr	Thr	Cys	Thr	Ser	Val	395	400	405
Gln	Glu	Gln	Gly	Leu	Lys	Ala	Asn	Asn	Ser	Lys	Glu	Leu	Ser	Met	410	415	420
Cys	Phe	Thr	Glu	Leu	Thr	Thr	Asn	Ile	Ile	Pro	Gly	Pro	Arg	Asp	425	430	435
Pro	Val	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	440	445	450
Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro	Lys	Pro	Lys	455	460	465
Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys	Val	Val	470	475	480
Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp	Tyr	485	490	495
Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu	500	505	510
Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	515	520	525
Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	530	535	540

-continued

Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
 545 550 555

Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
 560 565 570

Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
 575 580 585

Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
 590 595 600

Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu
 605 610 615

Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp
 620 625 630

Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
 635 640 645

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
 650 655 660

Ser Pro Gly Lys
 664

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1995 base pairs
 - (B) TYPE: Nucleic Acid
 - (C) STRANDEDNESS: Single
 - (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATGATCTTGG CAAACGCCTT CTGCCTCTTC TTCTTTTTTAG ACGAAACCCT 50

CCGCTCTTTG GCCAGCCCTT CCTCCCTGCA GGGCTCTGAG CTCCACGGCT 100

GGCGCCCCCA AGTGGACTGT GTCCGGGCCA ATGAGCTGTG TCGGGCTGAA 150

TCCAAC TGCA GCTCCAGGTA CCGCACCTT CGGCAGTGCC TGGCAGGCCG 200

GGATCGCAAT ACCATGCTGG CCAATAAGGA GTGCCAGGCA GCCCTGGAGG 250

TCTTGCAGGA AAGCCCACTG TATGACTGCC GCTGCAAGCG GGGCATGAAG 300

AAGGAGCTGC AGTGTCTGCA GATCTACTGG AGCATCCATC TGGGGCTGAC 350

AGAGGGTGAG GAGTTCTATG AAGCTTCCCC CTATGAGCCT GTGACCTCGC 400

GCCTCTCGGA CATCTTCAGG CTCGCTTCAA TCTTCTCAGG GACAGGGACA 450

GACCCGGCGG TCAGTACCAA AAGCAACCAC TGCCTGGATG CCGCCAAGGC 500

CTGCAACCTG AATGACAAC T GCAAGAAGCT TCGCTCCTCT TATATCTCCA 550

TCTGCAACCG TGAGATCTCT CCCACCGAAC GCTGCAACCG CCGCAAGTGC 600

CACAAGGCTC TGCGCCAGTT CTTTGACCGT GTGCCAGCG AGTATACCTA 650

CCGCATGCTC TTCTGCTCCT GTCAGGACCA GGCATGTGCT GAGCGTCGCC 700

GGCAAACCAT CCTGCCAGT TGCTCCTATG AGGACAAGGA GAAGCCCAAC 750

TGCCTGGACC TGCGCAGCCT GTGTCTGACA GACCACCTGT GCCGGTCCCG 800

ACTGGCAGAT TTCCACGCCA ACTGTCGAGC CTCCTACCGG ACAATCACCA 850

GCTGTCCTGC GGACAAC TAC CAGGCATGTC TGGGCTCCTA TGCTGGCATG 900

ATTGGGTTTG ATATGACACC CAACTATGTG GACTCCAACC CCACGGGCAT 950

CGTGGTGTCT CCCTGGTGCA ATTGTCGTGG CAGTGGGAAC ATGGAAGAAG 1000

AGTGTGAGAA GTTCTCAGG GACTTCACGG AAAACCCATG CCTCCGGAAT 1050

-continued

GCCATTCAGG CCTTTGGTAA TGGCACAGAT GTGAACATGT CTCCCAAAGG 1100
 CCCCTCACTC CCAGCTACCC AGGCCCTCG GGTGGAGAAG ACTCCTTCAC 1150
 TGCCAGATGA CCTCAGTGAC AGCACCAGCC TGGGGACCAG TGTCATCACC 1200
 ACCTGCACAT CTATCCAGGA GCAAGGGCTG AAGGCCAACA ACTCCAAAGA 1250
 GTTAAGCATG TGCTTCACAG AGCTCACGAC AAACATCAGT CCAGGGTCTA 1300
 GAGACCCGGT GGACAAAAC CACACATGCC CACCGTGCCC AGCACCTGAA 1350
 CTCCTGGGGG GACCGTCAGT CTTCTCTTC CCCCCAAAAC CCAAGGACAC 1400
 CCTCATGATC TCCCGGACCC CTGAGGTCAC ATGCGTGGTG GTGGACGTGA 1450
 GCCACGAAGA CCCTGAGGTC AAGTTCAACT GGTACGTGGA CGGCGTGGAG 1500
 GTGCATAATG CCAAGACAAA GCCGCGGGAG GAGCAGTACA ACAGCACGTA 1550
 CCGTGTGGTC AGCGTCCTCA CCGTCCTGCA CCAGGACTGG CTGAATGGCA 1600
 AGGAGTACAA GTGCAAGGTC TCCAACAAAG CCCTCCCAGC CCCCATCGAG 1650
 AAAACCATCT CCAAAGCCAA AGGGCAGCCC CGAGAACCAC AGGTGTACAC 1700
 CCTGCCCCCA TCCCGGAAG AGATGACCAA GAACCAGGTC AGCCTGACCT 1750
 GCCTGGTCAA AGGCTTCTAT CCCAGCGACA TCGCCGTGGA GTGGGAGAGC 1800
 AATGGGCAGC CGGAGAACAA CTACAAGACC ACGCCTCCCG TGCTGGACTC 1850
 CGACGGCTCC TTCTCCTCT ACAGCAAGCT CACCGTGGAC AAGAGCAGGT 1900
 GGCAGCAGGG GAACGTCTTC TCATGCTCCG TGATGCATGA GGCTCTGCAC 1950
 AACCCTACA CGCAGAAGAG CCTCTCCCTG TCTCCGGGTA AATGA 1995

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 664 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Ile Leu Ala Asn Ala Phe Cys Leu Phe Phe Phe Leu Asp Glu
 1 5 10 15
 Thr Leu Arg Ser Leu Ala Ser Pro Ser Ser Leu Gln Gly Ser Glu
 20 25 30
 Leu His Gly Trp Arg Pro Gln Val Asp Cys Val Arg Ala Asn Glu
 35 40 45
 Leu Cys Ala Ala Glu Ser Asn Cys Ser Ser Arg Tyr Arg Thr Leu
 50 55 60
 Arg Gln Cys Leu Ala Gly Arg Asp Arg Asn Thr Met Leu Ala Asn
 65 70 75
 Lys Glu Cys Gln Ala Ala Leu Glu Val Leu Gln Glu Ser Pro Leu
 80 85 90
 Tyr Asp Cys Arg Cys Lys Arg Gly Met Lys Lys Glu Leu Gln Cys
 95 100 105
 Leu Gln Ile Tyr Trp Ser Ile His Leu Gly Leu Thr Glu Gly Glu
 110 115 120
 Glu Phe Tyr Glu Ala Ser Pro Tyr Glu Pro Val Thr Ser Arg Leu
 125 130 135
 Ser Asp Ile Phe Arg Leu Ala Ser Ile Phe Ser Gly Thr Gly Thr
 140 145 150

-continued

545	550	555
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro		
560	565	570
Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu		
575	580	585
Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser		
590	595	600
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu		
605	610	615
Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp		
620	625	630
Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met		
635	640	645
His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu		
650	655	660
Ser Pro Gly Lys		
664		

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 670 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTGCAAGAAG CTGCGCTCCT CCTACATCTC CATCTGCAAC CGCGAGATCT	50
CGCCCACCGA GCGCTGCAAC CGCCGCAAGT GCCACAAGGC CCTGCGCCAG	100
TTCTTCGACC GGGTGCCCGAG CGAGTACACC TACCGCATGC TCTTCTGCTC	150
CTGCCAAGAC CAGGCGTGCG CTGAGCGCCG CCGGCAAACC ATCCTGCCCA	200
GCTGCTCCTA TGAGGACAAG GAGAAGCCCA ACTGCCTGGA CCTGCGTGGC	250
GTGTGCCGGA CTGACCACCT GTGTCGGTCC CGGCTGGCCG ACTTCCATGC	300
CAATTGTCGA GCCTCCTACC AGACGGTCAC CAGCTGCCCT GCGGACAATT	350
ACCAGGCGTG TCTGGGCTCT TATGCTGGCA TGATTGGGTT TGACATGACA	400
CCTAACTATG TGGACTCCAG CCCCACTGGC ATCGTGGTGT CCCCTGGTGC	450
AGCTGTCGTG GCAGCGGGAA CATGGAGGAG GAGTGTGAGA AGTTCCTCAG	500
GACTTCACCG AGAACCCATG CCTCCGGAAC GCCATCCAGG CCTTTGGCAA	550
CGGCACGGAC GTGAACGTGT CCCCAAAAGG CCCTCGTTCC AGGCCACCCA	600
GGCCCCTCGG GTGGAGAAGA CGCCTTCTTT GCCAGATGAC CTCAGTGACA	650
GTACCAGCTT GGGACCAGT	670

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2378 base pairs
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TTCTATCGAT TGAATCCCC GGGGATCCTC TAGAGATCCC TCGACCTCGA	50
CCCACGCGTC CGCCGGGCGG CGGCTTTGGA TTTTGGGGGG GCGGGGACCA	100

-continued

GCTGCGCGGC	GGCACCATGT	TCCTAGCCAC	TCTGTACTTC	GCGCTGCCAC	150
TCCTGGATTT	GCTGATGTCC	GCCGAGGTGA	GTGGTGGAGA	CCGTCTGGAC	200
TGTGTGAAAG	CCAGCGATCA	GTGCCTGAAAG	GAACAGAGCT	GCAGCACCAA	250
GTACCGCACA	CTAAGGCAGT	GCGTGGCGGG	CAAGGAAACC	AACTTCAGCC	300
TGACATCCGG	CCTTGAGGCC	AAGGATGAGT	GCCGTAGCGC	CATGGAGGCC	350
TTGAAGCAGA	AGTCTCTGTA	CAACTGCCGC	TGCAAGCGGG	GCATGAAGAA	400
AGAGAAGAAT	TGTCTGCGTA	TCTACTGGAG	CATGTACCAG	AGCCTGCAGG	450
GAAATGACCT	CCTGGAAGAT	TCCCCGTATG	AGCCGGTTAA	CAGCAGGTTG	500
TCAGATATAT	TCCGGGCAGT	CCCCTTCATA	TCAGATGTTT	TCCAGCAAGT	550
GGAACACATT	TCCAAAGGGA	ACAACCTGCC	GGACGCAGCC	AAGGCCTGCA	600
ACCTGGACGA	CACCTGTAAG	AAGTACAGGT	CGGCCTACAT	CACCCCCTGC	650
ACCACCAGCA	TGTCCAACGA	GGTCTGCAAC	CGCCGTAAGT	GCCACAAGGC	700
CCTCAGGCAG	TTCTTCGACA	AGGTTCCGGC	CAAGCACAGC	TACGGGATGC	750
TCTTCTGCTC	CTGCCGGGAC	ATCGCCTGCA	CCGAGCGGCG	GCGACAGACT	800
ATCGTCCCCG	TGTGCTCCTA	TGAAGAACGA	GAGAGGCCCA	ACTGCCTGAG	850
TCTGCAAGAC	TCCTGCAAGA	CCAATTATAT	CTGCAGATCT	CGCCTTGCA	900
ATTTTTTTTAC	CAACTGCCAG	CCAGAGTCAA	GGTCTGTCAG	CAACTGTCTT	950
AAGGAGAACT	ACGCAGACTG	CCTCCTGGCC	TACTCGGGAC	TGATTGGCAC	1000
AGTCATGACT	CCCAACTACG	TAGACTCCAG	CAGCCTCAGC	GTGGCACCAT	1050
GGTGTGACTG	CAGCAACAGC	GGCAATGACC	TGGAAGACTG	CTTGAAATTT	1100
CTGAATTTTT	TTAAGGACAA	TACTTGTCTC	AAAAATGCAA	TTCAAGCCTT	1150
TGGCAATGGC	TCAGATGTGA	CCATGTGGCA	GCCAGCCCCT	CCAGTCCAGA	1200
CCACCACTGC	CACCACTACC	ACTGCCTTCC	GGGTCAAGAA	CAAGCCTCTG	1250
GGGCCAGCAG	GGTCTGAGAA	TGAGATCCCC	ACACACGTTT	TACCACCCTG	1300
TGCGAATTTG	CAGGCTCAGA	AGCTGAAATC	CAATGTGTGCG	GGTAGCACAC	1350
ACCTCTGTCT	TTCTGATAGT	GATTTCCGAA	AGGATGGTCT	CGCTGGTGCC	1400
TCCAGCCACA	TAACCACAAA	ATCAATGGCT	GCTCCTCCCA	GCTGCAGTCT	1450
GAGCTCACTG	CCGGTGCTGA	TGCTCACCGC	CCTTGCTGCC	CTGTTATCTG	1500
TATCGTTGGC	AGAAACGTCG	TAGCTGCATC	CGGGAAAACA	GTATGAAAAG	1550
ACAAAAGAGA	ACCAAGTATT	CTGTCCCTGT	CCTCTTGAT	ATCTGAAAAT	1600
CCAGTTTTAA	AAGCTCCGTT	GAGAAGCAGT	TTCACCCAAC	TGGAACTCTT	1650
TCCTTGTTTT	TAAGAAAGCT	TGTGGCCCTC	AGGGGCTTCT	GTTGAAGAAC	1700
TGCTACAGGG	CTAATTCCAA	ACCCATAAGG	CTCTGGGGCG	TGGTGC GGCT	1750
TAAGGGGACC	ATTTGCACCA	TGTAAAGCAA	GCTGGGCTTA	TCATGTGTTT	1800
GATGGTGAGG	ATGGTAGTGG	TGATGATGAT	GGTAATTTTA	ACAGCTTGAA	1850
CCCTGTTCTC	TCTACTGGTT	AGGAACAGGA	GATACTATTG	ATAAAGATTC	1900
TTCCATGTCT	TACTCAGCAG	CATTGCCTTC	TGAAGACAGG	CCCGCAGCCT	1950
AGTGTGAATG	ACAAGTGGAG	GTTGGCCTCA	AGAGTGGACT	TGGCAGACTC	2000
TACCTTGTAG	TAATGTTTAC	CTTCCGTGT	ATGGTCTCCA	CAGAGTGTTT	2050

-continued

ATGTATTTAC AGACTGTTCT GTGATCCCCC AACAAACAACA ACCACAAATT	2100
CCTTGGTCAC CTCCAAATGT AACCGGTCCT TTAGCCCAGT AGAGGAGGGT	2150
GGGTGTGGCC CTGGCACAGC TCCCGGATTG TTGATGGGCA CTCTCCTGAG	2200
CTTTGCTTGA GTGAGAAGCT GAATGTAGCT GAAAATCAAC TCTTCTTACA	2250
CTTAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA	2300
AAAAAAAAAA AAAAGTTTA GGGATAACAG GGTAATGCGG CCGCGTCGAC	2350
CTGCAGAAGC TTGGCCGCCA TGGCCCAA	2378

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 468 amino acids
- (B) TYPE: Amino Acid
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Met Phe Leu Ala Thr Leu Tyr Phe Ala Leu Pro Leu Leu Asp Leu	1	5	10	15
Leu Met Ser Ala Glu Val Ser Gly Gly Asp Arg Leu Asp Cys Val	20	25	30	
Lys Ala Ser Asp Gln Cys Leu Lys Glu Gln Ser Cys Ser Thr Lys	35	40	45	
Tyr Arg Thr Leu Arg Gln Cys Val Ala Gly Lys Glu Thr Asn Phe	50	55	60	
Ser Leu Thr Ser Gly Leu Glu Ala Lys Asp Glu Cys Arg Ser Ala	65	70	75	
Met Glu Ala Leu Lys Gln Lys Ser Leu Tyr Asn Cys Arg Cys Lys	80	85	90	
Arg Gly Met Lys Lys Glu Lys Asn Cys Leu Arg Ile Tyr Trp Ser	95	100	105	
Met Tyr Gln Ser Leu Gln Gly Asn Asp Leu Leu Glu Asp Ser Pro	110	115	120	
Tyr Glu Pro Val Asn Ser Arg Leu Ser Asp Ile Phe Arg Ala Val	125	130	135	
Pro Phe Ile Ser Asp Val Phe Gln Gln Val Glu His Ile Ser Lys	140	145	150	
Gly Asn Asn Cys Leu Asp Ala Ala Lys Ala Cys Asn Leu Asp Asp	155	160	165	
Thr Cys Lys Lys Tyr Arg Ser Ala Tyr Ile Thr Pro Cys Thr Thr	170	175	180	
Ser Met Ser Asn Glu Val Cys Asn Arg Arg Lys Cys His Lys Ala	185	190	195	
Leu Arg Gln Phe Phe Asp Lys Val Pro Ala Lys His Ser Tyr Gly	200	205	210	
Met Leu Phe Cys Ser Cys Arg Asp Ile Ala Cys Thr Glu Arg Arg	215	220	225	
Arg Gln Thr Ile Val Pro Val Cys Ser Tyr Glu Glu Arg Glu Arg	230	235	240	
Pro Asn Cys Leu Ser Leu Gln Asp Ser Cys Lys Thr Asn Tyr Ile	245	250	255	
Cys Arg Ser Arg Leu Ala Asp Phe Phe Thr Asn Cys Gln Pro Glu	260	265	270	
Ser Arg Ser Val Ser Asn Cys Leu Lys Glu Asn Tyr Ala Asp Cys				

-continued

140				145				150						
Asp	Ala	Ala	Lys	Ala	Cys	Asn	Leu	Asp	Asp	Ile	Cys	Lys	Lys	Tyr
				155					160					165
Arg	Ser	Ala	Tyr	Ile	Thr	Pro	Cys	Thr	Thr	Ser	Val	Ser	Asn	Asp
				170					175					180
Val	Cys	Asn	Arg	Arg	Lys	Cys	His	Lys	Ala	Leu	Arg	Gln	Phe	Phe
				185					190					195
Asp	Lys	Val	Pro	Ala	Lys	His	Ser	Tyr	Gly	Met	Leu	Phe	Cys	Ser
				200					205					210
Cys	Arg	Asp	Ile	Ala	Cys	Thr	Glu	Arg	Arg	Arg	Gln	Thr	Ile	Val
				215					220					225
Pro	Val	Cys	Ser	Tyr	Glu	Glu	Arg	Glu	Lys	Pro	Asn	Cys	Leu	Asn
				230					235					240
Leu	Gln	Asp	Ser	Cys	Lys	Thr	Asn	Tyr	Ile	Cys	Arg	Ser	Arg	Leu
				245					250					255
Ala	Asp	Phe	Phe	Thr	Asn	Cys	Gln	Pro	Glu	Ser	Arg	Ser	Val	Ser
				260					265					270
Ser	Cys	Leu	Lys	Glu	Asn	Tyr	Ala	Asp	Cys	Leu	Leu	Ala	Tyr	Ser
				275					280					285
Gly	Leu	Ile	Gly	Thr	Val	Met	Thr	Pro	Asn	Tyr	Ile	Asp	Ser	Ser
				290					295					300
Ser	Leu	Ser	Val	Ala	Pro	Trp	Cys	Asp	Cys	Ser	Asn	Ser	Gly	Asn
				305					310					315
Asp	Leu	Glu	Glu	Cys	Leu	Lys	Phe	Leu	Asn	Phe	Phe	Lys	Asp	Asn
				320					325					330
Thr	Cys	Leu	Lys	Asn	Ala	Ile	Gln	Ala	Phe	Gly	Asn	Gly	Ser	Asp
				335					340					345
Val	Thr	Val	Trp	Gln	Pro	Ala	Phe	Pro	Val	Gln	Thr	Thr	Thr	Ala
				350					355					360
Thr	Thr	Thr	Thr	Ala	Leu	Arg	Val	Lys	Asn	Lys	Pro	Leu	Gly	Pro
				365					370					375
Ala	Gly	Ser	Glu	Asn	Glu	Ile	Pro	Thr	His	Val	Leu	Pro	Pro	Cys
				380					385					390
Ala	Asn	Leu	Gln	Ala	Gln	Lys	Leu	Lys	Ser	Asn	Val	Ser	Gly	Asn
				395					400					405
Thr	His	Leu	Cys	Ile	Ser	Asn	Gly	Asn	Tyr	Glu	Lys	Glu	Gly	Leu
				410					415					420
Gly	Ala	Ser	Ser	His	Ile	Thr	Thr	Lys	Ser	Met	Ala	Ala	Pro	Pro
				425					430					435
Ser	Cys	Gly	Leu	Ser	Pro	Leu	Leu	Val	Leu	Val	Val	Thr	Ala	Leu
				440					445					450
Ser	Thr	Leu	Leu	Ser	Leu	Thr	Glu	Thr	Ser					
				455					460					

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

His Gln Asn Leu Ser Asp Gly Lys
 1 5 8

-continued

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

His Gln Asn Ile Ser Asp Gly Lys
 1 5 8

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 7 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

His Gln Ser Leu Gly Thr Gln
 1 5 7

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 8 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Val Ile Ser Ser His Leu Gly Gln
 1 5 8

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 11 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

Pro Lys Asn Ser Ser Met Ile Ser Asn Thr Pro
 1 5 10 11

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 10 amino acids
 (B) TYPE: Amino Acid
 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

Asp Lys Thr His Thr Cys Pro Pro Cys Pro
 1 5 10

55

What is claimed is:

1. An isolated nucleic acid molecule comprising a nucleic acid sequence encoding a neurturin-binding polypeptide comprising an NTN α sequence selected from the group consisting of:

- (a) mature NTN α extracellular domain amino acid sequence from amino acid 23 to amino acid 431 of SEQ ID NO: 3 or SEQ ID NO:6;
 (b) a naturally-occurring mammalian homolog of (a) having at least 90% amino acid sequence identity to (a);
 (c) an amino acid sequence having at least 95% identity to (a); and

(d) an amino acid sequence of (a) having a deletion of one to thirty amino acids.

2. The isolated nucleic acid molecule of claim 1, wherein the neurturin-binding polypeptide comprises the mature human NTN α extracellular domain amino acid sequence from amino acid 23 to amino acid 431 of SEQ ID NO: 3.

3. The isolated nucleic acid molecule according to claim 1 encoding a chimeric NTN α wherein the NTN α sequence is fused to an immunoglobulin heavy chain constant domain sequence.

4. The isolated nucleic acid molecule of claim 1, further comprising a promoter operably linked to the nucleic acid molecule.

5. An expression vector comprising the nucleic acid molecule of claim 1 operably linked to control sequences recognized by a host cell transformed with the vector.

6. An in vitro host cell comprising the vector of claim 5.

7. A process of using a nucleic acid molecule encoding NTN α to effect production of NTN α , comprising culturing the host cell of claim 6 under conditions allowing expression of NTN α .

8. The process of claim 7 further comprising recovering the NTN α from the host cell culture.

9. An isolated polynucleotide comprising (a) a nucleotide sequence encoding a polypeptide comprising the amino acid sequence from amino acid 23 to amino acid 431 of SEQ ID NO: 3, or (b) a nucleotide sequence fully complementary to the nucleotide sequence of (a).

10. The isolated polynucleotide of claim 9, wherein the amino acid sequence comprises SEQ ID NO 3.

11. The isolated polynucleotide of claim 9, comprising the nucleic acid sequence of SEQ ID NO 2 that encodes amino acid 23 to amino acid 431 of SEQ ID NO 3.

12. The isolated polynucleotide of claim 11 comprising SEQ ID NO 2.

13. An expression vector comprising the polynucleotide of claim 9.

14. An in vitro host cell comprising the expression vector of claim 13.

15. A method for producing a polypeptide comprising the amino acid sequence from amino acid 23 to amino acid 431 of SEQ ID NO:3, the method comprising the steps of:

a) culturing the host cell of claim 14 under conditions suitable for the expression of the polypeptide; and

b) recovering the polypeptide from the host cell culture.

16. An isolated polynucleotide, comprising a nucleotide sequence that hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence fully complementary to the nucleotide sequence shown in SEQ ID NO: 2, and encodes an NTN α capable of binding NTN with a K_d of 10 pM or below.

17. The isolated polynucleotide of claim 16 that encodes a naturally-occurring polypeptide.

18. The isolated polynucleotide of claim 16 that encodes a soluble polypeptide.

19. The isolated polynucleotide of claim 16 that encodes a GPI-linked membrane protein.

20. An expression vector comprising the polynucleotide of claim 16.

21. An in vitro host cell comprising the expression vector of claim 20.

22. An isolated polynucleotide comprising (a) a nucleotide sequence encoding a polypeptide comprising the

amino acid sequence from amino acid 23 to amino acid 431 of SEQ ID NO: 6, or (b) a nucleotide sequence fully complementary the nucleotide sequence of (a).

23. The isolated polynucleotide of claim 22, wherein the polypeptide comprises SEQ ID NO 6.

24. The isolated polynucleotide of claim 22 comprising the nucleic acid sequence of SEQ ID NO 5 that encodes amino acid 23 to amino acid 431 of SEQ ID NO 6.

25. The isolated polynucleotide of claim 24 comprising SEQ ID NO 5.

26. An expression vector comprising the polynucleotide of claim 22.

27. An in vitro host cell comprising the expression vector of claim 26.

28. A method for producing a polypeptide comprising the amino acid sequence from amino acid 23 to amino acid 431 of SEQ ID NO:5, the method comprising the steps of:

a) culturing the host cell of claim 27 under conditions suitable for the expression of the polypeptide; and

b) recovering the polypeptide from the host cell culture.

29. An isolated polynucleotide, comprising a nucleotide sequence that hybridizes under stringent conditions to a polynucleotide having a nucleotide sequence fully complementary to the nucleotide sequence shown in SEQ ID NO: 5, and encodes an NTN α capable of binding NTN with a K_d of 10 pM or below.

30. The isolated polynucleotide of claim 29 that encodes a naturally-occurring polypeptide.

31. The isolated polynucleotide of claim 29 that encodes a soluble polypeptide.

32. The isolated polynucleotide of claim 29 that encodes a GPI-linked membrane protein.

33. An expression vector comprising the polynucleotide of claim 29.

34. An in vitro host cell comprising the expression vector of claim 33.

35. The host cell of any one of claims 14, 21, 27 and 34, which is an insect cell or a mammalian cell.

36. The isolated polynucleotide of claim 16, which does not hybridize to the complement of a polynucleotide encoding GDNFR α under the same stringent hybridization conditions.

37. The isolated polynucleotide of claim 29, which does not hybridize to the complement of a polynucleotide encoding GDNFR α under the same stringent hybridization conditions.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,372,453 B1
DATED : April 16, 2002
INVENTOR(S) : Robert D. Klein et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,

Item [73], Assignee, instead of “[73] **Genetech, Inc.**, South San Francisco, CA (US)”
should read -- [73] **Genentech, Inc.**, South San Francisco, CA (US) --;

Signed and Sealed this

Twenty-third Day of July, 2002

Attest:

A handwritten signature in black ink, appearing to read "James E. Rogan", written over a horizontal line.

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office